08/453,350

Atty Dkt 22300-20054.01
PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of:

CARL-HENRIK HELDIN et al.

Serial No.: 07/574,540 Group Art Unit: 185

Filing Date: 27 August 1990 Examiner: M. Porta

Title: RECOMBINANT DNA ENCODING PDGF A-CHAIN POLYPEPTIDES

RULE 131 DECLARATION

The Honorable Commissioner of
Patents and Trademarks
Washington, D.C. 20231

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[redacted].02

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Date Jan 10, 1992

CARL-HENRIK HELDIN

Date Jan 9-1992

CHRISTER BETSHOLTZ

Date Jan 19-1992

BENGT WESTERMARK

Date _____

TIMOTHY J. KNOTT

Date _____

JAMES SCOTT

Date _____

GRAEME I. BELL

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Date _____ _____
BENGT WESTERMARK

Date 16th Jan 1992 Timothy J. Knott
TIMOTHY J. KNOTT

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cDNA sequence and chromosomal localization of human platelet-derived growth factor A-chain and its expression in tumour cell lines

Christer Betsholtz*, Ann Johnsson†, Carl-Henrik Heldin†**, Bengt Westermark†, Peter Lind‡**, Mickey S. Urdea‖, Roger Eddy¶, Thomas B. Shows¶, Karen Philpott‖, Andrew L. Mellor‖, Timothy J. Knott* & James Scott*

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The amino-acid sequence of the precursor of the human tumour cell line-derived platelet-derived growth factor (PDGF) A-chain has been deduced from complementary DNA clones and the gene localized to chromosome 7. The protein shows extensive homology to the PDGF B-chain precursor. Expression of the PDGF A-chain gene is independent of that of the PDGF B-chain in a number of human tumour cell lines, and secretion of a PDGF-like growth factor of relative molecular mass 31,000 correlates with expression of A- but not B-chain messenger RNA.

HUMAN platelet-derived growth factor (PDGF) consists of dimers of homologous polypeptide chains, denoted A and B (refs 1, 2). Whether PDGF is a heterodimer or a mixture of homodimers is not known, but the dimer structure is functionally important, since reduction irreversibly destroys the biological activity of PDGF. Connective tissue-derived cells display high-affinity cell-surface receptors for PDGF and respond to PDGF by receptor autophosphorylation, tyrosine phosphorylation of cytoplasmic substrates, increased cytoplasmic calcium concentration, activation of protein kinase C, cytoplasmic alkalinization, reorganization of actin filaments, specific gene expression and DNA synthesis (reviewed in ref. 3).

The B-chain precursor is encoded by the c-*sis* gene, the cellular counterpart to the transforming gene v-*sis* of simian sarcoma virus (SSV)[4-6]. The human c-*sis* gene has been mapped to the long arm of chromosome 22 (ref. 7) and has been shown to be transcribed in several human tumour cell lines[8-11] as well as in certain normal cell types such as endothelial cells[12], placental cytotrophoblasts[13] and activated macrophages[14,15].

The primary translation product of the v-*sis* gene undergoes dimerization and proteolytic processing at the N- and C-terminals, yielding a product of relative molecular mass (M_r) 24,000 (24K) which resembles a dimer of PDGF B-chains and is recognized by anti-PDGF antibodies[16]. There is ample evidence that SSV-induced transformation is mediated by a PDGF-like growth factor. First, SSV-transformed cells contain and release a PDGF agonist activity[17-22]. Second, acutely SSV-transformed human fibroblasts are morphologically indistinguishable from PDGF-stimulated cells, and more significantly, their transformed phenotype is reverted by the addition of anti-PDGF antibodies to the culture medium[23]. Studies of the transforming protein of SSV have indicated that assembled PDGF B-chains alone form an active mitogen. Furthermore, amino-acid sequence analysis of porcine PDGF has revealed that this dimeric factor contains only one type of chain, corresponding to the human B-chain[24].

Evidence that homodimers of PDGF A-chains also have biological activity was recently obtained from studies of a PDGF-like mitogen released from a human osteosarcoma cell line, U-2 OS. This factor[25], which binds to the PDGF receptor, was found to be a homodimer of a polypeptide chain that displays a chemical fragmentation pattern, chromatographic behaviour and N-terminal amino-acid sequence identical to that of the PDGF A-chain[26].

We report here the complete primary structure of the PDGF A-chain precursor deduced from its complementary DNA sequence, its structural relation to the PDGF B-chain precursor, the chromosomal localization of the gene and its expression in human tumour cell lines. We also present data showing that the release of biologically active 31K PDGF-like growth factors by human tumour cell lines correlates with PDGF A-chain but not B-chain gene expression.

PDGF A-chain cDNA

A λgt10 cDNA library was constructed using poly(A)$^+$ RNA purified from the human clonal glioma cell line U-343 MGaC12:6. This cell line was chosen because it produces higher quantities of PDGF receptor competing activity than do other cell lines investigated. An 36-base-pair (bp) oligonucleotide probe (PDGF-A-1) corresponding to the N-terminus of the PDGF A-chain amino-acid sequence (Fig. 1) was synthesized and used to screen the library (2×10^6 recombinant clones) at low stringency. Of 48 positive clones, 4 hybridized to a 37-bp oligonucleotide probe (PDGF-A-2) directed against a mid-portion of the A-chain amino-acid sequence and were selected for further analysis.

DNA sequence analysis showed that the four clones overlapped and contained inserts of 800–1,400 bp (not shown). The complete nucleotide sequence, determined from one clone (D1), is shown in Fig. 1. The longest open reading frame of this 1.3-kilobase (kb) cDNA predicts a PDGF A-chain precursor protein of 211 amino acids (M_r ~23,000), and an in-frame termination codon is situated 81 bp upstream of the putative translation initiation site. Two additional ATG triplets lie within the 337 bp of the 5′-untranslated region sequenced, but these do not conform to the consensus for translation initiation[27] and predict only short polypeptides.

** Present addresses: Ludwig Institute for Cancer Research, Biomedicum, S-751 23 Uppsala, Sweden (C.-H.H.); KabiGen AB, Strandbergsgatan 49, S-112 87 Stockholm, Sweden (P.L.).

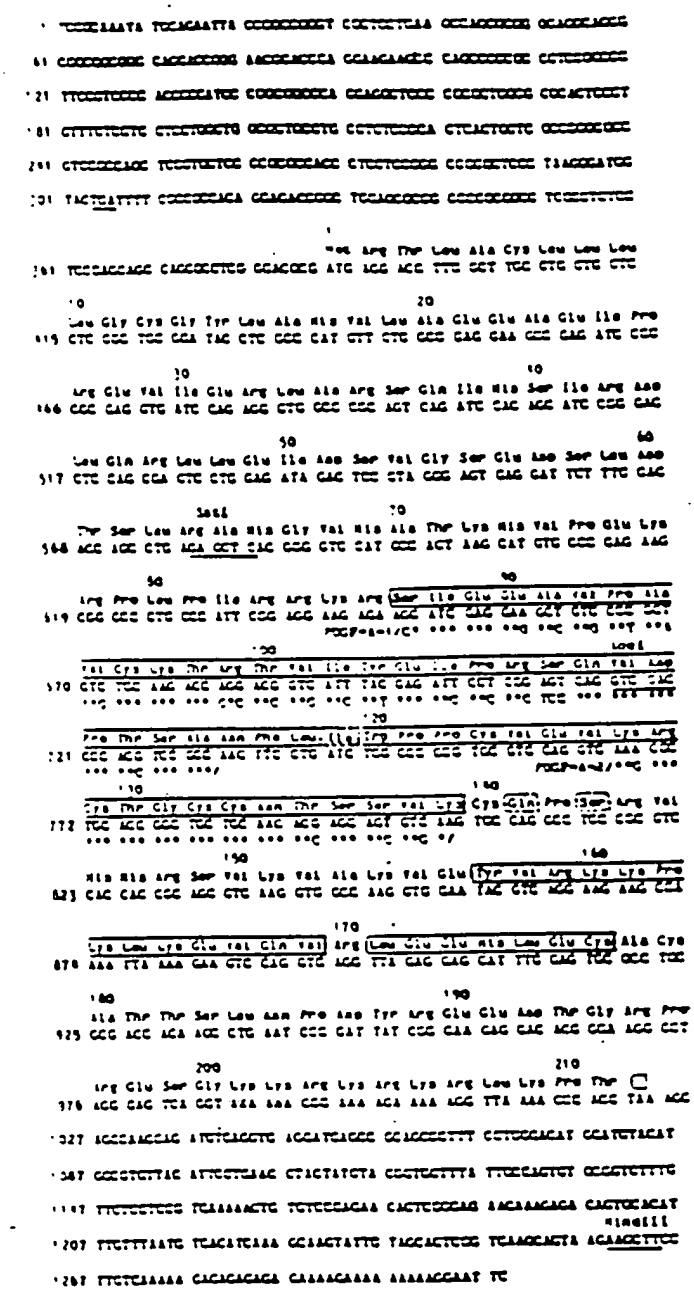

Fig. 1 Nucleotide sequence and deduced amino-acid sequence of the PDGF A-chain determined from a 1.3-kb cDNA clone (D1). An in-frame termination codon in the 5′-untranslated region is underlined. The PDGF A-chain cDNA encodes a 211-amino-acid precursor. Confirmed stretches of PDGF A-chain amino-acid sequence (from ref. 2) are boxed and differences indicated with dashed lines. Restriction endonuclease recognition sites used in the sequencing procedure are indicated. The sequences at which the two oligonucleotide probes PDGF-A-1 and PDGF-A-2 used to identify PDGF A-chain cDNAs were directed are indicated: * implies identity to the cDNA sequence. Box indicates termination codon.

Methods. Standard molecular biology techniques were used where not otherwise indicated. The double-stranded DNA probe PDGF-A-1 was synthesized as two overlapping 50-bp oligonucleotides and radiolabelled using [α-^{32}P]-deoxynucleoside triphosphates and the Klenow fragment of DNA polymerase I. PDGF-A-2 was synthesized as a 37-base template and a 12-base complementary primer and was radiolabelled as PDGF-A-1. Both oligonucleotides were synthesized using solid-phase phosphoramidite methodology[31]. The human clonal glioma cell line U-343.MGaC12:6 was the source of poly(A)$^+$ RNA, which was prepared using the LiCl/urea method modified as described elsewhere[11]. Oligo(dT)-primed synthesis of double-stranded cDNA was performed according to Gubler and Hoffman[32]. The resulting cDNA was treated with T4 DNA polymerase (Biolabs) and subcloned into *Eco*RI-cleaved λgt10 using *Eco*RI linkers. The recombinant phage were plated on *Escherichia coli* C600 hfl. Duplicate nitrocellulose filter lifts were hybridized with ^{32}P-labelled oligonucleotide probes at 42 °C in 20% formamide, 5 × SSC, 50 mM sodium phosphate pH 7.0, 5 × Denhardt's, 0.1% SDS, 200 μg ml^{-1} sonicated salmon sperm DNA and washed in 0.5 × SSC, 0.1% SDS at the same temperature. The nucleotide sequence of the PDGF A-chain cDNA restriction fragments was determined by dideoxynucleotide chain termination after subcloning into M13 phage derivatives.

The protein sequence matches that derived by amino-acid sequencing of the PDGF A-chain[2] except at amino acids 119, 141 and 143, found to be Ile, Gln and Ser, respectively, instead of the previously assigned Val, Arg and Thr (Fig. 1). These discrepancies could be due to protein sequencing errors. Alternatively, as the cDNA was obtained from a tumour cell line, it is possible that the sequence deviates from that of the normal PDGF A-chain transcript. The ATG codon at position 388 precedes a basic amino acid (Arg) followed by 18 hydrophobic residues (Fig. 1). This is characteristic of a signal peptide sequence and is consistent with the observation that PDGF A-chain homodimers produced by human osteosarcoma cells are secreted[23,26]. Comparison with preferred signal peptidase cleavage sites[28] suggests that processing may occur between amino acids Ala 20 and Glu 21. The N-terminal sequence of platelet PDGF A-chain is found at amino acid 87, indicating that a propeptide of 66 amino acids (44% charged residues) is cleaved from the precursor to generate a 125-amino-acid A-chain protein. This cleavage occurs after a run of four basic amino acids, Arg-Arg-Lys-Arg. Additional proteolytic processing may occur in the C-terminal region.

Human PDGF is heterogeneous in relative molecular ma probably reflecting proteolytic cleavage in the platelets or deg dation during the purification procedure. SDS-gel el trophoresis of the two constituent chains has revealed that variability is confined mainly to the A-chain[3,4]. As amino-a sequencing showed a unique A-chain N-terminus[3,4], heterogeneity may arise through proteolysis in the highly b C-terminus (Fig. 1). After N-terminal modification, the A-ch would have a M_r of ~14,000, although the highest-M_r form the A-chain migrates as a 16–18K species on SDS gels[3,4]. discrepancy may be due t glycosylation and/or the anomal migration commonly observed for canonic proteins. A sir consensus sequence f r asparagine-linked glycosylation (Asn Ser/Thr) is found at position 134–136, consistent with the re that PDGF contains carb hydrate[29]. The mature B-chain c n t possess any *N*-glycosylation sites, although ne is pre in the N-terminal propeptide (Fig. 2).

The 5′-untranslated region of the PDGF A-chain messe RNA has a high G+C content (~75%) and a high propor of CpG dinucleotides. CpG-rich regions are found at the 5′ of many vertebrate genes and may indicate that these reg

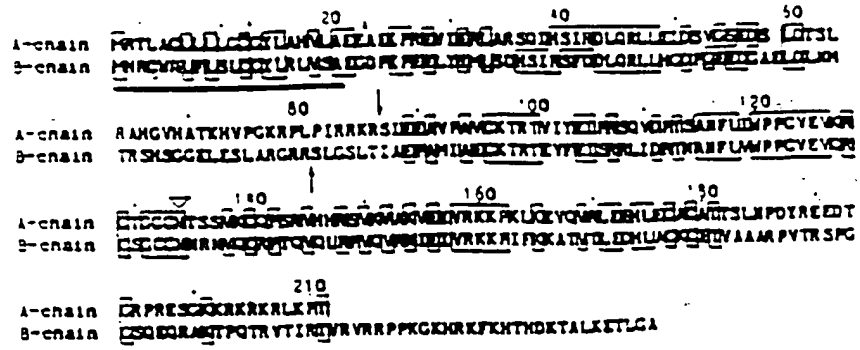

Fig. 2 Alignment and comparison of the two PDGF chain precursor amino-acid sequences. Homologies are boxed. Cysteine residues are shaded. Signal sequences are underlined and *N*-glycosylation sites are marked with a ▽. N-terminal processing sites are marked with arrows.

are protected from methylation[30]. Clone D1 carries 281 bp of 3'-untranslated sequence ending with a $(dGA)_6$ repeat followed by a short poly(A) stretch and *Eco*RI linker but no recognizable polyadenylation signal. Of four cDNA clones sequenced, three terminate around this same position but a fourth contains a longer dGA repeat, extends 370 bp farther 3', but also lacks a polyadenylation signal and poly(A) tail (not shown). It is possible that the three similar clones, including D1, are primed internally on an oligo(A) stretch and represent a mRNA spliced differently from the clone with the longer 3' extension, a possibility in agreement with the presence of multiple A-chain transcripts (Fig. 3). The exact relationship between the different clones and mRNAs remains unknown, although cDNAs in which bases 968–1,036 (Fig. 1) are deleted have been identified (data not shown) and are believed to be the result of differential splicing. If translated, these clones predict an A-chain precursor 15 residues smaller and lacking the basic C-terminal region.

Relationship with the PDGF B-chain

Comparison of the amino-acid sequences of the PDGF A- and B-chain precursors shows them to be similar in size, with an overall amino-acid sequence homology of 40% after insertion of several gaps in their N-terminal portions. A significantly higher degree of homology is seen in a region within the mature chains: amino acids 89–181 of the A-chain is 56% homologous to the B-chain (Fig. 2). Notably, all eight cysteine residues are conserved within the mature chains, implying a similar tertiary structure. Accordingly, homodimers of either the B- or A-chain can bind to the PDGF receptor. The basic region Val-Arg-Lys-Lys-Pro (amino acids 158–162) may be relevant in this context, since basic polypeptides such as protamine sulphate and polylysine have been shown to compete with ^{125}I-PDGF for binding to the PDGF receptor[31].

A significant degree of homology is also seen between part of the N-terminal propeptide sequences, particularly a 10-amino-acid stretch at position 39–48 in the A-chain precursor. The analogous region in v-*sis* is not essential for this gene's transforming function[32]. In addition, apparently identical 24K B-chain dimers were formed in NIH 3T3 cells transfected with v-*sis* constructs with or without the N-terminal propeptide region[32]. Thus, it is difficult to assign a role for this region in post-translational processing of the two PDGF chains.

While there is essentially no sequence homology between the precursor C-terminal sequences, both contain a high proportion of basic amino-acid residues (Fig. 2). Significant nucleotide sequence homology between the A- and B-chain transcripts is observed only in those regions where the amino-acid sequence is strongly conserved.

Hydrophobicity plots (data not shown) indicate that the A- and B-chain precursors are hydrophilic proteins with two major conserved hydrophobic domains. The first corresponds to the signal sequences, while the second is located 28 and 34 residues from the N-terminus of the processed A- and B-chain respectively (Fig. 2) and coincides with a 12-amino-acid conserved region in which there is only one difference (Ile/Val) between the two proteins.

Chromosomal localization

Using 36 human–mouse somatic cell hybrids, we mapped the PDGF A-chain gene to the pter→q22 region of chromosome 7 (Table 1). No other growth factor genes have been localized to this chromosome. The PDGF B-chain gene (c-*sis*) has been mapped[7] to the long arm of chromosome 22. Interestingly, after duplication of the ancestral PDGF gene, the A- and B-chain genes have acquired different chromosomal localizations.

PDGF mRNA expression in tumour cells

Northern blot hybridization analysis using poly(A)$^+$ RNA from various human cell types shows that the PDGF A-chain mRNA is expressed in several of the transformed cell lines examined but is not found in normal human fibroblasts or freshly isolated

Table 1 Distribution of the PDGF A-chain gene with human chromosomes in human–mouse cell hybrids

		Human chromosome																					
PDGF/Chrom.		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X
No. of concordant hybrids	(+/+)	9	12	13	12	11	10	19	12	5	16	13	13	12	16	12	8	17	12	9	12	15	8	10
	(−/−)	19	15	8	13	10	15	18	9	17	9	14	10	12	9	13	15	6	8	18	8	3	12	7
No. of discordant hybrids	(+/−)	9	7	7	8	9	10	0	8	15	4	6	7	8	4	7	12	3	8	11	8	5	11	6
	(−/+)	0	4	8	6	9	4	0	10	1	10	5	9	7	10	6	4	12	11	1	11	16	6	10
	% Discordancy	24	29	42	36	46	36	0	46	42	36	29	41	38	36	34	41	39	49	31	49	54	46	48

A PDGF A-chain cDNA probe (clone D1) was hybridized to Southern blots containing *Eco*RI- or *Hin*dIII-digested DNA from human–mouse hybrids. Presence of the human PDGF A-chain gene in the hybrids was determined by scoring the presence or absence of human bands on the blots. The first symbol in the parentheses indicates hybrids that were either positive (+) or negative (−) for the PDGF A-chain gene, while the second symbol indicates hybrids that either contained (+) or lacked (−) the particular chromosome. Concordant hybrids have either retained or lost the PDGF A-chain gene together with a specific human chromosome. Discordant hybrids either retained the gene, but not a specific chromosome, or the reverse. Per cent discordancy indicates the degree of discordant segregation of the PDGF A-chain gene and a chromosome. A 0% discordancy is the basis for chromosome assignment. One hybrid, JSR-17S, with a 7/9 translocation, indicates that the PDGF A-chain gene is localized to the pter→q22 region of chromosome 7. The table is compiled from 39 cell hybrids involving 14 unrelated human cell lines and 4 mouse cell lines[11,12]. The hybrids were characterized by chromosome analysis, by mapped enzyme markers and partly by mapped DNA probes.

Table 2 Compared data on the expression of the PDGF A- and B-chain genes and secretion of PDGF-like growth factors by human tumour cell lines and normal cells

Cell line	B-chain mRNA	A-chain mRNA	Secretion of a 31K PDGF-like protein	PDGF-receptor competing activity (ng ml^{-1})	Mitogenic activity in conditioned medium inhibitable by PDGF antibodies
Tumour cells					
U-2 OS	−	++	++	10	−
U-4 SS	++	++	++	10	−
U-393 OS*	−	+	−	3	ND
SAOS-II	−	+	−	0	ND
SKLMS	−	+	−	2	ND
B-5 GT	−	++	++	15	−
B-6 FS	−	+	−	2	ND
RD	−	++	++	25	−
U-343 MGaC12:6	++	+++	+++	40	−
U-563 MG*	−	−	−	0	ND
Normal cells					
AG 1523	−	−	−	0	−
Macrophages	−	−	ND	ND	ND

The cell lines have the following origins: U-2 OS, osteosarcoma[43]; U-4 SS, synovial sarcoma[43]; U-393 OS, osteosarcoma; SAOS-II, osteosarcoma[44] SKLMS, leiomyosarcoma[44]; B-5 GT, giant cell sarcoma[43]; B-6 FS, fibrosarcoma[43]; RD, rhabdomyosarcoma[44]; U-343 MGaC12:6, glioma[47]; U-563 MG, glioma; AG 1523, a human foreskin fibroblast line obtained from the Human Genetic Mutant Cell Repository, Camden, New Jersey. Macrophage RNA was prepared from freshly isolated peritoneal macrophages, collected by centrifugation of dialysis fluid (1,500g, 5 min). +/− indicate presence/absence of hybridizing mRNAs on Northern blots or specifically immunoprecipitated 31K proteins that become converted to 16.5–17K species on reduction. PDGF receptor competing activity of serum-free tumour cell-conditioned medium was measured as inhibition of the binding of added ^{125}I-labelled PDGF to human foreskin fibroblasts[22,23,48]. Using a standard curve constructed from results obtained with pure unlabelled PDGF (5–200 ng ml^{-1}), PDGF receptor competing activity of the samples was converted to PDGF equivalents (ng ml^{-1}). Determination of mitogenic activity in serum-free tumour cell-conditioned medium was performed as described previously[49] in the absence or presence of 50 μg ml^{-1} affinity-purified anti-PDGF IgG[50]. ND, not determined.

* Unpublished cell lines of Department of Pathology, Uppsala, Sweden.

peritoneal macrophages (Fig. 3). The macrophages we used were not activated *in vitro* before RNA preparation; after activation, macrophages have been found to express c-*sis* and produce a PDGF-like growth factor[14,15]. All positive cell lines display three major hybridizing bands, corresponding to transcripts of 1.9, 2.3 and 2.8 kb. Certain human tumour cell lines have been reported to express the PDGF B-chain (c-*sis*) transcript[3–11]. Some of the cell lines investigated here, such as the glioma U-343 MGaC12:6 and the osteosarcoma line U-2 OS, express both types of transcripts, whereas other cell lines, such as the rhabdomyosarcoma RD and the giant cell sarcoma B-5 GT, express only the A-chain mRNA, and the glioma U-563 MG, like normal fibroblasts and macrophages, expresses none. The A- and B-chain genes are thus regulated independently in human tumour cell lines examined.

Secretion of PDGF-like growth factors

The synthesis of PDGF-like growth factors by human tumour cell lines has been extensively reported[3–11,20,22,23,28,33–35]. These factors are all 31K proteins, split by reduction into two closely migrating 16.5K and 17K bands (Fig. 4)[10,22,23]. They possess the biological features of PDGF and are recognized by anti-PDGF antibodies. Our data show that immunoprecipitation of PDGF-like proteins from the conditioned medium of the human tumour cell lines studied correlates with the expression of PDGF A-chain but not B-chain mRNA (Table 2). This suggests that all of the PDGF-like factors detected by anti-PDGF antibodies in the medium of these human tumour cell lines are composed of only PDGF A-chains, despite the fact that some express both A- and B-chain mRNA (Fig. 3). This view is supported by the detailed structural characterization of the 31K factor secreted by U-2 OS cells which showed it to be an A-chain homodimer[28].

Discussion

Our study shows that the two constituent chains of human PDGF are encoded by genes located on different chromosomes, and that both genes can be expressed independently in human tumour cell lines.

PDGF is stored in the platelet α-granules and released in conjunction with the platelet release reaction (reviewed in ref. 1). It is believed to act as a mitogen for connective tissue cells at the site of vascular injury. Homodimers of both PDGF A- and B-chains possess PDGF receptor agonist activity. What, therefore, is the significance of the presence of both types of chains in human PDGF? Studies of the B-chain homodimer encoded by v-*sis* and the osteosarcoma-derived A-chain homodimer have revealed differences in the efficiency of secretion and/or affin-

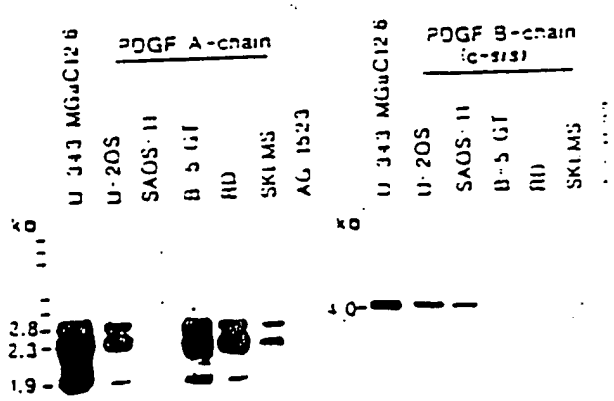

Fig. 3 Northern blot analysis of poly(A)$^+$ RNA (10 μg per lane) from various normal and neoplastic human cells. The origins of the cell lines are given in Table 2 legend. Cells growing in monolayer were collected at confluency. Total cellular RNA was prepared and selected once on oligo(dT)-cellulose (Pharmacia). Agarose gel electrophoresis, blotting to nitrocellulose and hybridization to ^{32}P-labelled PDGF A-chain cDNA (left) or PDGF B-chain (c-*sis*) cDNA (right) were performed as described previously. Filters were exposed to Kodak XAR-5 films at −70 °C for 4 days.

Fig. 4 Immunoprecipitation of metabolically labelled PDGF-like growth factors produced by human tumour cells. The origins of the cell lines are given Table 2 legend. Confluent 850-cm² roller bottle cultures of cells were labelled with ^{35}S-cysteine (NEN; 600 Ci mmol^{-1}) as described elsewhere[3]. Briefly, cultures were pulsed with 250 μCi of ^{35}S-cysteine in 3 ml of cysteine-free medium for 3 h, and then chased in 3 ml of cysteine-containing medium for an additional 3 h. Media were pooled and sequentially precipitated with a control rabbit serum (*a*) and PDGF antiserum (*b*). Immunoprecipitates were absorbed to protein A-Sepharose (Pharmacia) and analysed on 13–18% SDS-polyacrylamide gels under reducing or non-reducing conditions. Dried gels were exposed to Kodak XAR-5 film for 4–7 days at −70 °C.

for a specific cellular compartment[22,24], raising the possibility that structural differences between the two chains serve different functions in relation to storage, release and association with the plasma membrane, extracellular matrix and plasma proteins[17–19]. For example, both types of homodimer are biologically active but their affinity for the PDGF receptor may differ both from each other and from the putative heterodimer. In fact, platelet PDGF appears to be more potent than the PDGF-like factors purified from human tumour cell line-conditioned media (C.-H.H. *et al.*, unpublished results). Furthermore, in spite of evidence that the transforming function of SSV is exerted by an externalized v-*sis* product, no accumulation of PDGF antigenic activity is seen in the medium of acutely SSV-transformed human fibroblast cultures, and anti-PDGF antibodies precipitate only low-*M*$_r$ monomers from the medium of SSV-transformed cells[23]. Apparently, after being released, the v-*sis* product remains associated with, or rapidly associates with, structures in the cell membrane including the PDGF receptor[13–16]. The low-*M*$_r$ monomers probably represent degraded v-*sis* products. In contrast, intact 31K A-chain homodimers can be immunoprecipitated from human tumour cell-conditioned medium. The A-chain may therefore contribute to the stability of PDGF.

The exact nature of the human PDGF subunit composition and the significance of the presence of both A- and B-chains remain unknown. The genetic basis for A-chain expression in human tumour cells is also unknown, as is its role in tumour growth. Several non-transformed cell types, endothelial cells[12], cytotrophoblasts[13], smooth muscle cells[20] and activated macrophages[4,13] have been shown to express the c-*sis* gene and/or to release PDGF-like growth factors. It will be interesting to see whether the A-chain gene is expressed in these normal cells, and to determine the subunit structure of the secreted factors. Knowledge of the PDGF A-chain precursor structure and access to PDGF A-chain cDNAs as molecular probes will certainly contribute to the elucidation of such matters.

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EXHIBIT A

From Page No.

DATE: [redacted]

GIVEN: 2 clones containing the human platelet-derived growth factor a-chain cDNA

"D1" plasmid ~1300-1500bp (EcoR

"13-1" λ clone slightly smaller than 1300 bp. (EcoRI)

PLAN: ISOLATE EcoRI FRAGMENTS FROM EACH OF THESE CLONES FOR SEQUENCING

"D1" PLASMID "pUC13-D1 1mg/ml"

V_T = 500λ

MIX: 200λ - EcoRI mix *
100λ - DNA in TE (100μg)
180λ - H_2O
20λ - EcoRI (NewEng and Biolabs)
lot 17() 20 UNITS/λ

REACT: 37°C, 12:55pm -

TEST: 2λ + 3λ - H_2O + 2λ - EcoRI stop on Δ GEL (1% AGAROSE)

* FINAL BUFFER: 100 mM Tris, pH 7.5 / 50mM NaCl / 5 mM MgCl / 100μg/ml BSA / 0.1% β-mercaptoE

To Page No

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Invented by

Recorded by

Dat [redacted]

Exhibit B
1 of 2

TLE _____ F N. _____

From Page No. —

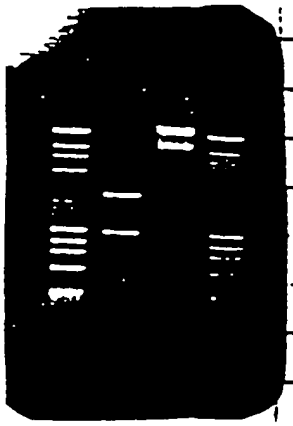
"13-1" λ clone

V_T = 200λ

MIX: 80.0λ – EcoRI mix
30.0λ ~~37.5λ~~ – DNA in TE (15μg)
85.0λ ~~77.5λ~~ – H_2O
5.0λ – EcoRI (NEB, lot 17, 20 U/

REACT: 37°C, 12:55pm –

TEST: 4λ + 2λ – STOP EcoRI on Δ gel



both DIGESTIONS complete

prors → "DI PLASMID"
2×50λ
~~2.5λ 50.0λ~~ – TE
(15.0λ – EcoRI)

λ 13-1
50λ – TE
(15λ – EcoRI)

Store @ –20°C [Mixture]

prep gel: 1% Agarose
135 mA

cut out FRGS → cut and pass through needles (16 GA, 18 GA, 20 GA, 22 GA) rt, o/n.
in lo-salt elutip-D buffer

T Page No

Exhibit B

Invented by

Dat

t No._____

TITLE _____ Book No._____

From Page N 38

WAIT FOR MORE DNA (GBC1),
~~LIGATE INTO VECTOR~~
CUT W/ ECORI
LIGATE MIXTURE INTO THE VECTOR.

pHPDGFA-103 ~~USABLE CLONES~~: (D1/E1 + pSV7d

correct clones: 2, 5, 7
we still don't know orientation.
cut w/ BamHI + HindIII.
(will be done by Anne O.).

D1

pUC13 - plasmid into HB101

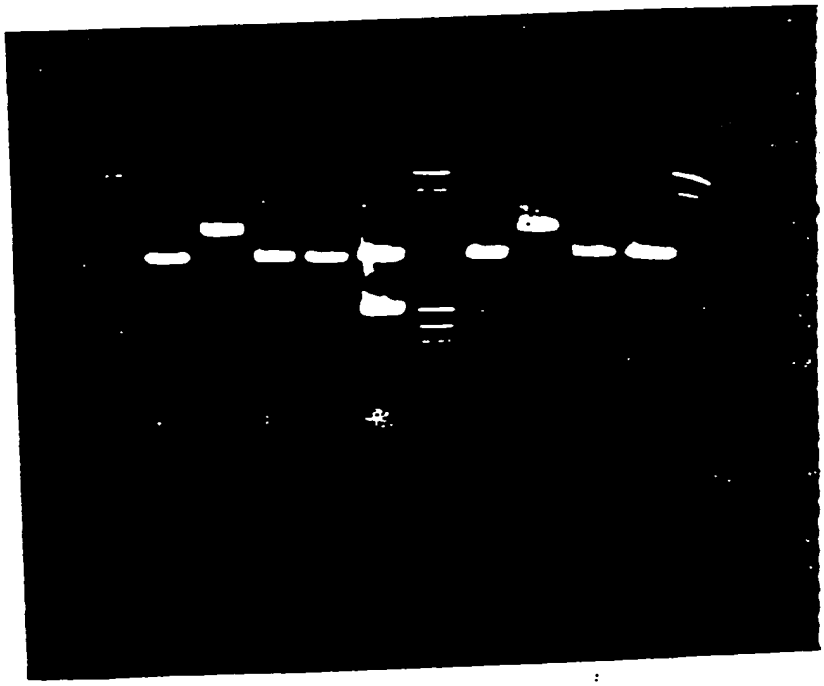
we got it → glycerol stock made

These clones cut w/ BamHI + HindIII →

#2, #7 are the correct orientation.

Make glycerol stocks of both

→ LB, scale DNA preps



To Pa

Witnessed & Understood by me. Date

Invented by

Recorded by

Date [redacted]

LIGATION TO expression VECTOR.

VECTOR: DSV7d / ECORI / -PO4
[redacted] LR
EST. 100 ng/λ ... 2.423 kb
Dilute 2λ INTO 8λ-H2O

DNA: DI - 1300 bp ~~4~~ ng/λ
λ13-1 - 600 bp ≤ 20 ng/9λ
λ13-1 - 1300 bp ≤ 20 ng/9λ

Cf = 4 μg/ml

FRAGMENT : VECTOR RATIO 2:1

λ13-1 // 600bp ~~Cf~~ Cf = 2 μg/λ → 20.0λ

mix: 9.0λ - 600 bp - λ13-1 DNA
2.0λ - 20 ng/λ VECTOR
2.0λ - 10x KIN. B.
2.0λ - 10 mM ATP
4.0λ - H2O
+ 1.5λ - T4 Lig (NEB, 400U/λ lot 26)

λ13-1 // 1300bp Cf = 2 μg/λ → 20.0λ

mix: 9.0λ - 1300 bp - λ13-1 DNA
2.0λ - 20 ng/λ VECTOR
2.0λ - 10x KIN. B.
2.0λ - 10 mM ATP
5.0λ - H2O
+ 1.5λ - T4 LIG (11)

Exhibit C
1 of 4

To Page N

Witnessed & Understood by me, | Date | Witnessed by | Date [redacted]

Pr ject N.
Bo k N
TITLE hPDGF-a-chain

From Pag No.

DI (1300) // vector

~~C_f / ... / V_f 25λ~~

~~... 2.5λ ... ...~~

C_f = 3 μg/ml V_f = 20λ

8λ - DNA (32 ng)
1.5λ - vector (30 ng)
[2.0λ - 10 mM ATP
[2.0λ - 10x Lig. B
5.0λ - H_2O
+ 1.5λ - T4 Lig (")

react: 14°C, o/n   [... [redacted]]

DATE: [redacted]

~~...~~ prob → 92.5λ - H_2O

ADD: 7.5λ - 1M $CaCl_2$ (fresh)
200.0λ - competent HB101 E. coli

60' @ 4°C
1.5' @ 42°C

ADD: 3.0 ml - Luria Broth
37°C, 1 hr w/ agitation air shaker

Exhibit C
2 of 4

To Page No.

Witnessed & Understood by me,   Date   Invented by   Date [redacted]
Recorded by

Project No. _____ TITLE ECORI TRANSFORMANTS phPDGFA-102&3

Book No. _____

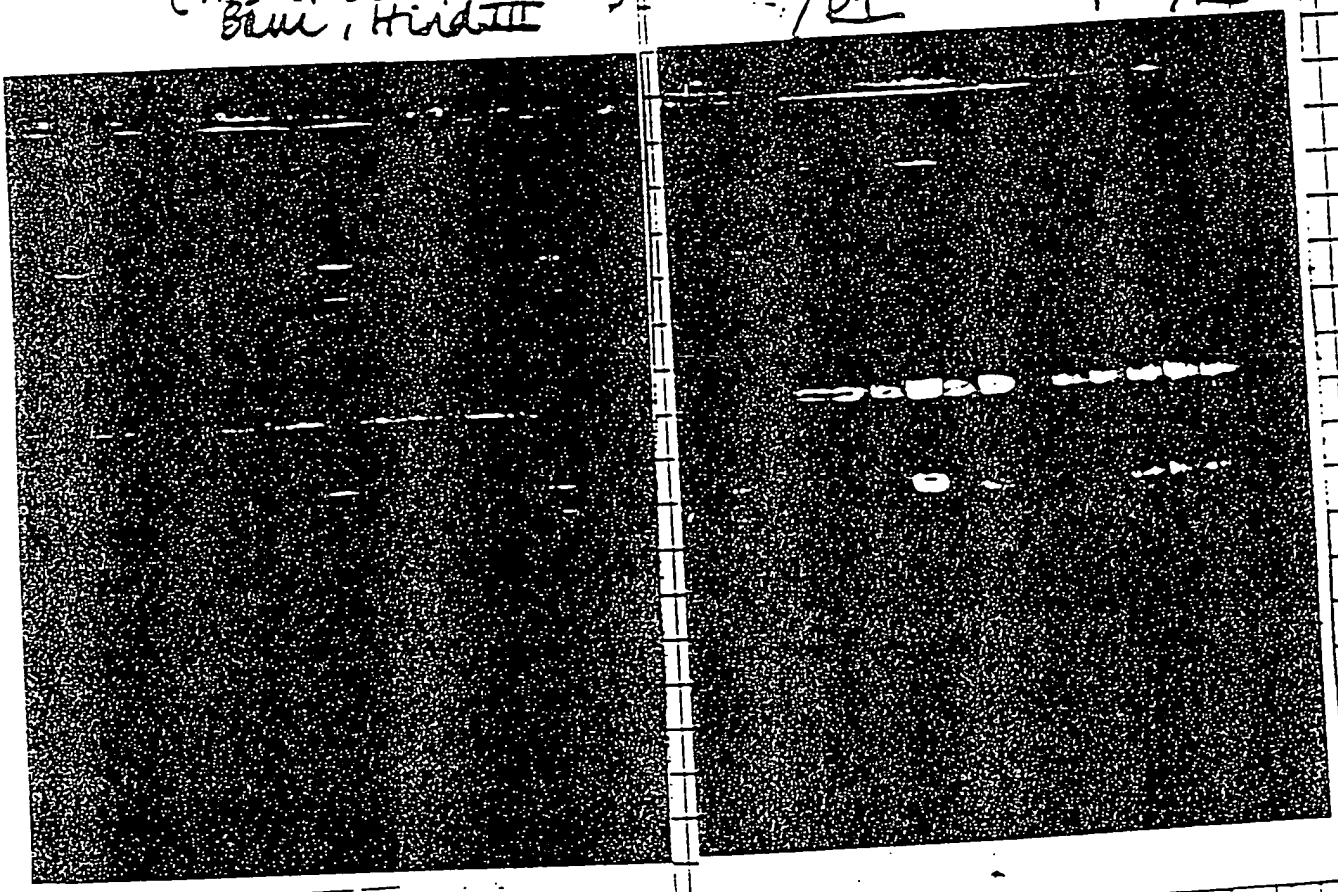
38

From Page No. _____

phPDGFA-102
(λ13-11 ~~ECORI~~ BamH, HindIII)

~~phPDGFA-103~~ phPDGFA-103
~~ECORI~~ / RI

pUC13-
plasmid
/RI



UPDATE: [redacted]

GELS RUN BY Anne:

phPDGFA-102 : (λ13-11 /RI → 130 a +600)

ATTEMPT TO CLONE THIS

NO CLONES.

To Pag No.

Witnessed & Understood by me. Date

Invented by

Recorded by

Date [redacted]

A. Animal cells. EcoRI fragments which contain the entire protein coding sequence for th A-chain and B-chain precursors were cloned into pSV7d (Figures 4, 5 and 6). CHO cells were transfected with each of these together with the amplifiable dihydrofolate reductase containing plasmid, pAD cell lines were isolated, grown up and assayed for PDGF activity using a radioreceptor competition assay which has a range of 10-100 ng/ml PDGF. Several of the cell lines transfected with the B-chain construct, pSV7d-PDGF-B1 (Figure 4), secrete 25-50 ng/ml PDGF/24h. Similar results were obtained with the A-chain construct, pSV7d-PDGF-A102(13-1) (Figure 5), which corresponds to the cDNA encoding the 196 amino acid precursor.

B. Saccharomyces cerevisiae. A synthetic gene coding for the mature B-chain was cloned into the ADH2-GAPDH promoter α-factor leader vector (Figure 7). Similarly after in vitro mutagenesis to generate XbaI and SalI sites at the ends of the two different mature A-chains, these fragments were also cloned into the above yeast expression vector. Yeast transformed with

these plasmids would be expected t synthesize a protein containing an NH_2-terminal α-fact r leader and COOH-terminal PDGF chain separated by a Lys-Arg (Figures 8, 9 and 10). Since this molecule is targated for secretion cleavage of the Lys-Arg by the yeast should result in secretion of the mature growth factor.

08/453,350

8

EGULAR UTILITY

PTO-436
8/78)

705175	PATENT DATE	PATENT NUMBER

L NUMBER	FILING DATE	CLASS	SUBCLASS	GROUP ART UNIT	EXAMINER
705,175	12/25/85	435		127	

K J. MURRAY, KING COUNTY, WA; JAMES D. KELLY, KING COUNTY, WA.

NTINUING DATA**********
IFIED THIS APPLN IS A CIP OF 06/660,496 10/12/84

IGN/PCT APPLICATIONS**********
IED

FILING LICENSE GRANTED 05/21/85 ***** SMALL ENTITY *****

claimed ☐ yes ☐ no nditions met ☐ yes ☐ no knowledged Examiner's Initials	AS FILED	STATE OR COUNTRY	SHEETS DRWGS.	TOTAL CLAIMS	INDEP. CLAIMS	FILING FEE RECEIVED	ATTORNEY'S DOCKET NO.
	⟹	CA	13	54	14	$ 535.00	5905410

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E SIS OF BIOLOGICALLY ACTIVE DEOXY ANALOGS IN EUCARYOTIC CELLS

This is to certify that annexed hereto is a true copy from the records of the United States Patent and Trademark Office of the application as originally filed which is identified above.

By authority of the
COMMISSIONER OF PATENTS AND TRADEMARKS

E. K. Jenkins

Certifying Officer

Description

EXPRESSION OF BIOLOGICALLY ACTIVE PDGF ANALOGS IN EUCARYOTIC CELLS

Technical Field

The present invention relates to the production of PDGF analogs in general, and more specifically, to the expression of biologically active PDGF analogs in eucaryotes.

Background Art

Human platelet derived growth factor (PDGF) has been shown to be the major mitogenic protein in serum for mesenchymal derived cells. This is well documented by numerous studies of platelet extracts or purified PDGF induction of either cell multiplication or DNA synthesis (a prerequisite for cell division) in cultured smooth muscle cells, fibroblasts and glial cells (Ross et al., PNAS 71: 1207, 1974; Kohler and Lipton, Exp. Cell Res. 87: 297,1947; Westermark and Wasteson, Exp. Cell Res. 98: 170, 1976; Heldin et al., J. Cell Phyiol. 105: 235, 1980; Raines and Ross, J. Biol. Chem 257: 5154, 1982). Furthermore, PDGF is a potent chemoattractant for cells that are responsive to it as a mitogen (Grotendorst et al., J. Cell Phyiol. 113: 261, 1982; Seppa et al., J. Cell Biol. 92: 584, 1982). It is not generally the case that mitogens also act as chemotactic agents. Due to its mitogenic activity, PDGF is useful as an important component of a defined medium for the growth of mammalian cells in culture, making it a valuable research reagent with multiple applications in the study of animal cell biology.

In vivo, PDGF normally circulates stored in the alpha granules of platelets. Injury to arterial endothelial linings causes platelets to adhere to the exposed connec-

tive tissue and release their granules. The released PDGF is thought to chemotactically attract fibroblasts and smooth muscle cells to the site of injury and to induce their focal proliferation as part of the process of wound repair (Ross and Glomset, N. England Journal of Medicine 295: 369, 1976).

It has been postulated that as a part of this response to injury, PDGF released by platelets may play a causative role in the development of the proliferative lesions of atherosclerosis (Ross and Glomset, ibid.) which is one of the principal causes of myocardial and cerebral infarction. Strategies for the prophylaxis and treatment of atherogenesis in the past have been narrowly directed toward reducing risk factors for the disease, such as lowering blood pressure in hypertensive subjects and reducing elevated cholesterol levels in hypercholesterolemic subjects.

Recent studies have shown that one of the two protein chains comprising PDGF and the putative transforming protein of simian sarcoma virus (SSV), an acute transforming retrovirus, appear to have arisen from the same or closely related cellular genes. In particular, computer analysis of a partial amino acid sequence of PDGF has revealed extensive homology with the gene product, p28sis, of SSV (Doolittle, Waterfield and Johnson, ibid.). Further, more recent studies have illustrated that p28sis and PDGF show antigenic as well as structural similarities (Robbins et al., Nature 305: 605, 1983; Niman, Nature 307: 180, 1984).

Although previous attempts, such as that summarized in Devare et al., (Cell 36: 43, 1984), have been made to express the v-sis gene in a transformed microorganism, they have not been successful in producing mitogenic material. More recently, investigators have described the production of p28sis in E. coli as a fusion protein. (Wang et al., J. Biol. Chem 259: 10645, 1984).

This protein appears to compete with PDGF for binding to PDGF receptor sites. While SSV transformed rodent cells have been shown to exhibit a mitogenic activity similar to PDGF (Deuel, et al., Science 221: 1348, 1983; Owen, et al., Science 225: 54, 1984), it is not clear that this activity is due to a gene product from SSV (i.e., $p28^{sis}$). Furthermore, cells transformed by a variety of viruses other than SSV produce a PDGF-like mitogen into the culture medium (Bowen-Pope et al., PNAS 81: 2396, 1984).

While natural PDGF may be isolated from human plasma or platelets as starting material, it is a complex and expensive process, in part due to the limited availability of the starting material. In addition, it is difficult to purify PDGF with high yield from other serum components due to its extremely low abundance and biochemical properties. Furthermore, the therapeutic use of products derived from human blood carries the risk of disease transmission due to contamination by, for example, hepatitis virus, cytomegalovirus, or the causative agent of Acquired Immune Deficiency Syndrome (AIDS).

In view of PDGF's clinical applicability in the treatment of injuries in which healing requires the proliferation of fibroblasts or smooth muscle cells and its value as an important component of a defined medium for the growth of mammalian cells in culture, the production of useful quantities of protein molecules similar to authentic PDGF which possess mitogenic activity is clearly invaluable.

In addition, the ability to produce relatively large amounts of PDGF would be a useful tool for elucidating the putative role of the v-sis protein, $p28^{sis}$, in the neoplastic process.

Further, since local accumulation of smooth muscle cells in the intamal layer of an arterial wall is central to the development of atherosclerotic lesions (Ross and Glomset, ibid.), one strategy for the prophylaxis and treatment of atherosclerosis would be to suppress smooth

muscle cell proliferation. The ability to produce large amounts of PDGF would be useful in developing inhibitors or designing specific approaches which prevent or interfere with the in vivo activity of PDGF in individuals with atherosclerosis.

Disclosure of The Invention

Briefly stated, the present invention discloses a DNA construct capable of directing the expression and secretion of biologically active PDGF analogs in eucaryotic cells. The DNA construct contains a transcriptional promoter followed downstream by a gene encoding a protein having substantially the same structure and/or mitogenic activity as PDGF, and a signal sequence capable of directing the secretion of the protein from the eucaryotic cell. The gene may be the v-sis gene or a derivative of the v-sis gene of simian sarcoma virus or portions thereof which encode a protein having biological activity. Further, the derivative of the v-sis gene may be the portion of v-sis gene which is substantially homologous to the B chain of PDGF. In addition, the gene may be the human cDNA gene for PDGF or portions thereof encoding a protein having biological activity.

Another aspect of the invention discloses a method of preparing biologically active PDGF analogs by introducing into a eucaryotic host a DNA construct capable of directing the expression and secretion of biologically active PDGF analogs in eucaryotic cells. The DNA construct contains a transcriptional promoter followed downstream by a gene encoding a protein having substantially the same structure and/or mitogenic activity as PDGF and a signal sequence capable of directing the secretion of the protein from the eucaryotic cell. Subsequent to introducing the DNA construct into the eucaryotic host, the method includes growing the eucaryotic host in an appropriate medium and then isolating the protein product of the gene from the

eucaryotic host. Eucaryotic host cells transformed with such a DNA construct are also disclosed.

The present invention further provides a method for promoting the growth of mammalian cells through incubating the cells with a biologically active PDGF analog expressed by a eucaryotic cell transformed with a DNA construct capable of directing the expression and secretion of biologically active PDGF analogs in eucaryotic cells. The DNA construct contains a transcriptional promoter followed downstream by a gene encoding a protein having substantially the same structure and/or mitogenic activity as PDGF and a signal sequence capable of directing the secretion of the protein from the eucaryotic cell.

In one embodiment of the invention, the eucaryotic cell may be a yeast cell, and the DNA construct more appropriately termed an extrachromosomal element.

Other aspects of the invention will become evident upon reference to the following detailed description and attached drawings.

Brief Description of The Drawings

Figure 1A is a schematic restriction map of the proviral genome of SSV.

Figure 1B depicts the nucleotide sequence and predicted amino acid sequence encoded by the v-sis region of SSV genome.

Figure 2 illustrates the construction of a plasmid which contains the MF 1 promoter and secretory signal sequence upstream of the v-sis gene.

Figure 3 illustrates the construction of plasmid p192.

Figure 4 illustrates the oligonucleotide directed deletion mutagenesis of the amino terminal sixty-six v-sis codons.

Figure 5 illustrates the construction of plasmid p270.

Figure 6 illustrates the insertion of v-sis expression units upstream of the TPI terminator.

Figure 7 illustrates the replacement of the M..α1 promoter with the TPI promoter and inclusion of the VS2α construction in the pCPOT vector.

Figure 8 illustrates the construction of plasmid pTVS2αT.

Figure 9 illustrates the constuction of a B chain expression unit VSB and its introduction into the pMPOT vector.

Figure 10 depicts the electrophoretic and subsequent hybridization analysis of total RNA isolated from a yeast host transformed with various plasmids probed with a nick-translated v-sis gene fragment.

Figure 11 depicts the results of ELISA of concentrated culture media from the yeast transformants containing plasmids pVSα, pVS2α, pll7-2 and pCPOT.

Figure 12 is a dose response curve of mitogenic activity of concentrated culture media from yeast transformants containing plasmids pVSα and pll7-2, compared to purified PDGF.

Figure 13 is a dose response curve of PDGF receptor binding by media concentrates from yeast transformants containing plasmids pVSαm, PVS2αm, pVSBm and pMPOT2 compared to authentic PDGF.

Figure 14 is a dose response curve of mitogenic activity of media concentrates from yeast transformants containing plasmids pVSαm, pVS2αm, pVSBM, and pMPOT2 compared to authentic PDGF.

Best Mode For Carrying Out the Invention

Prior to setting forth the invention, it may be helpful to an understanding thereof to set forth definitions of certain terms to be used hereinafter.

Polypeptide: A polymer of amino acids.

Reading Frame: The arrangement of nucleotide codons which encode an uninterrupted stretch of amino acids. During translation of an mRNA, the proper reading frame must be maintained. For example, the sequence GCUGGUUGUAAG may be translated into three reading frames or phases, depending on whether one starts with G, with C, or with U, and thus may yield three different peptide products. Translation of the template begins with an AUG codon, continues with codons for specific amino acids, and terminates with one of the translation termination codons.

Coding Sequence: DNA sequences which in the appropriate reading frame directly code for the amino acids of a protein.

Complementary DNA: or cDNA. A DNA molecule or sequence which has been enzymatically synthesized from the sequences present in an mRNA template.

Secretory Signal Sequence: That portion of a gene encoding a signal peptide. A signal peptide is the amino acid sequence in a secretory protein which signals its translocation into the secretory pathway of the cell. Signal peptides generally occur at the beginning (amino terminus) of the protein and are 20-40 amino acids long with a stretch of 9-10 hydrophobic amino acids in their center. Very often the signal sequence is proteolytically cleaved from the protein during the process of secretion.

Cell Surface Receptor: A protein molecule at the surface of a cell which specifically interacts with or binds a molecule approaching the cell's surface. Once the receptor has bound the cognate molecule, it effects specific changes in the physiology of the cell.

Mitogen: A molecule which stimulates cells to undergo mitosis. Mitosis is asexual somatic cell division leading to two daughter cells, each having the same number of chromosomes as the parent cell.

Transformation: The process of stably and hereditably altering the genotype of a recipient cell or microorganism by the introduction of purified DNA. This is typically detected by a change in the phenotype of the recipient organism.

Transcription: The process of producing mRNA template from a structural gene.

Expression: The process, starting with a structural gene, of producing its polypeptide, being a combination of transcription and translation. An expression vector is a plasmid derived construction designed to enable the expression of a gene carried on the vector.

Plasmid: An extrachromosomal double stranded DNA sequence comprising an intact "replicon" such that the plasmid is replicated in a host cell. When the plasmid is placed within a unicellular organism, the characteristics of that organism may be changed or transformed as a result of the expression of the DNA sequences of the plasmid. For example, a plasmid carrying the gene for tetracycline resistance (tet^R) transforms a cell previously sensitive to tetracycline into one which is resistant to it.

Yeast Promoter: DNA sequences upstream from a yeast gene which promotes its transcription.

Biological Activity: Some function or set of activities performed by a molecule in a biological context (i.e., in an organism or an *in vitro* facsimile). In the

case of PDGF, these biological activities include binding to cell surface receptor molecules, inducing chemotaxsis and inducing mitogenesis of responsive cell types.

As noted above, human platelet derived growth factor (PDGF) has been shown to be a major mitogenic protein in serum. PDGF is known to be composed of two polypeptide chains, an A chain and a B chain, which are held together by disulfide bonds to form the biologically active molecule. The A chain and B chain alone do not appear to exhibit any mitogenic activity, (Raines and Ross, ibid.) and attempts to reconstitute activity by reoxidation of the reduced polypeptides have not been successful. Recently, the amino acid sequence of the B chain has been shown to be substantially homologous to a portion of the v-sis gene product, $p28^{sis}$ (Doolitte et al., Science 221: 275, 1983; Waterfield et al., Nature 304: 35, 1984; and Johnson et al., Embo 3: 921, 1984). The homology between these two proteins strongly suggests that they are derived from the same or closely related cellular genes.

Given the fact that the B chain alone is not biologically active and that previous attempts directed toward expressing v-sis sequences in E. coli did not yield mitogenic material, it would not be expected that merely expressing a portion of the v-sis gene homologous to a portion of the PDGF gene in a microorganism would result in a molecule which exhibited mitogenic activity. The present invention however, unlike the previous attempts noted above, was designed to express the v-sis gene or portions thereof absent of heterologous sequences, such that the expressed molecules are more closely related to the B chain of PDGF. Further, the expression system of the present invention was designed to produce the gene product via a eucaryotic secretory pathway. This enables the expressed protein molecules to be properly processed and assembled such that they exhibit biological activity. Indeed, the

present invention, in contrast to previous efforts, results in the secretion of PDGF analogs which are biologically active.

In its active form, PDGF is a heat stable protein composed of heterogeneously sized species of between 28,000 and 31,000 Daltons, all of the individual species being active in and stimulating DNA synthesis (Raines and Ross, ibid.; Deuel et al., J. Biol. Chem. 256: 8896, 1981; Antoniades, PNAS 78: 7314, 1981). Where individual species withmolecular weights of 27,000; 28,500; 29,000; and 31,000 Daltons have been isolated and assayed, they have been found to have comparable mitogenic activity and amino acid composition (Raines and Ross, ibid.) Further, these species show extensive tryptic peptide homology. The slight variations in size among the species are most probably due to differences in carbohydrate composition and proteolysis.

Through studies of PDGF which has been extensively purified from platelet-rich human plasma, it is likely, as noted above, that PDGF is composed of two polypeptide chains, an A chain (14,000 Daltons) and a B chain (16,000 Daltons), which are disulfide bonded together to form the biologically active dimer molecule (Raines & Ross, Deuel et al., Antoniades, ibid.). The PDGF nomenclature found in the literature is not consistent (Doolittle et al., Waterfield et al., Raines and Ross, Johnsson et al., ibid.). The nomenclature of Johnsson et al., ibid.) wherein the two polypeptides found in pure PDGF are called A chain and B chain. The B chain is homologous to p28^{sis} and was previously called "peptide I" (Waterfield et al., ibid.) or "1a" (Doolittle et al., ibid.). The A chain was previously termed "peptide II" (Waterfield et al., ibid.) or "2a" (Doolittle et al.,ibid.). Data derived from a partial amino acid sequence of PDGF indicate that the two polypeptide chains (A chain and B chain) show some homology (Doolittle et al., ibid., Waterfield et al., ibid., and

Johnsson et al., ibid., Antoniades and Hunkapiller, Science 220: 963, 1983). The A chain and B chain alone do not appear to exhibit any mitogenic activity, and attempts to reconstitute activity by reoxidation of the reduced polypeptides have not been successful (Raines & Ross, ibid.).

The v-sis gene, as mentioned above, is the transforming gene of simian sarcoma virus (SSV). The v-sis gene has been cloned and its DNA sequence determined (Devare et al., PNAS 79: 3179, 1982; Devare et al., PNAS 80: 731, 1983). Analysis of this sequence revealed an open reading frame which could encode a 28,000 Dalton protein, designated $p28^{sis}$. Subsequently, such a protein was identified in SSV infected cells (Niman, ibid.; Robbins, ibid.). The predicted amino acid sequence of the v-sis gene product, $p28^{sis}$, was found to have a high degree of homology with the actual amino acid sequence of a portion of the B chain of PDGF (Johnsson, ibid.). The homology of the PDGF B chain to the v-sis gene product begins at amino acid 67 of $p28^{sis}$, a serine, and continues for approximately 109 amino acids to a threonine residue at amino acid 175. The amino acid sequences preceding and following the B chain homologous region of $p28^{sis}$ are not homologous to either the A or B chains of mature PDGF (Johnsson, ibid.) In addition, PDGF and $p28^{sis}$ have been shown to be similar antigenically (Niman, ibid.; Robbins, ibid.). The v-sis gene product, $p28^{sis}$, a protein of approximately 225 amino acids, appears to be proteolytically processed to a protein of approximately 20,000 Daltons ($p20^{sis}$) in SSV infected cells (Niman, ibid.; Robbins, ibid.). This 20,000 Dalton protein can be immunoprecipitated with antiserum against PDGF.

As noted above, previous attempts at expressing v-sis sequences in prokaryotes did not yield biologically active material. Further, the v-sis gene product $p28^{sis}$, as well as PDGF itself, are secreted mammalian proteins. In order to achieve biologically active material, the

present invention utilizes the secretory pathway of eucaryotic cells to express the v-sis gene and derivatives of the v-sis gene. Expression and secretion of the v-sis gene product from a eucaryotic cell enables processing and assembly which results in molecules with native and active conformation.

The secretory pathways of most eucaryotes are believed to be similar. In particular, mammalian cell and yeast cell secretory pathways are well characterized and are homologous. The presence of a secretory signal sequence on the expressed polypeptide is an important element in eucaryotes, due to its role in introducing the molecule into the secretory pathway, thereby leading to proper assembling and processing. Provided that appropriate transcriptional promoter and secretory signal sequences are utilized, generally any eucaryote could express and secrete the v-sis gene product in a biologically active form.

An easily manipulable and well characterized eucaryote is the yeast cell. For these reasons, yeast was chosen as a model example of an appropriate eucaryotic cell within the present invention. Accordingly, the v-sis gene and fragments thereof encoding the 109 amino acids with homology to the PDGF B chain were inserted into yeast extrachromosomal elements containing a yeast promoter capable of directing the expression of biologically active PDGF analogs. In accordance with the present invention, the yeast promoter is followed downstream by a fragment of the v-sis gene which encodes a protein having substantially the same structure and/or mitogenic activity as PDGF.

Genes which encode a protein having substantially the same structure and/or mitogenic activity as PDGF include the v-sis gene or a derivative of the v-sis gene of simian sarcoma virus (SSV) or portions thereof or the human cDNA gene for PDGF or portions thereof. Specifically, DNA sequences encoding polypeptides substantially homologous to

the B chain of PDGF are preferred. The genes to be utilized in the extrachromosomal element may be isolated using standard recombinant DNA techniques.

The human PDGF cDNA gene may be isolated from a human cDNA library made from an appropriate source of messenger RNA by using the v-sis gene or a fragment thereof as a hybridization probe. A preferred source of mRNA is human umbilical vein endothelial cells. These cells can be cultured *in vitro* for short periods of time and are known to secrete PDGF into the culture medium (DiCorleto and Bowen-Pope, PNAS 80: 1919, 1983). The identity of this cDNA gene as that encoding PDGF may be verified by DNA sequencing.

Promoters which may be utilized in yeast include the yeast alpha-factor (MFα1) promoter and the yeast triose phosphate isomerase (TPI) promoter. Promoters may also be obtained from other yeast genes, e.g., Alcohol Dehydrogenase 1 (ADH1), Alcohol Dehydrogenase 2 (ADH2).

The constructions described herein were designed such that the v-sis gene product would be secreted from the yeast cell into the media. This was accomplished through use of the secretion signal sequence of the yeast mating pheromone alpha-factor (Kurjan and Herskowitz, Cell 30: 933, 1982; Julius et al., Cell 36: 309, 1984; and Brake et al., PNAS 81: 4642, 1984) although other secretion signals may be used. To ensure the efficient transcription termination and polyadenylation of mRNA, a yeast terminator sequence, such as the triose phosphate isomerase terminator, was added. (Alber and Kawasaki, J. Molec. Genet. Appl. 1: 419, 1982.)

Once an appropriate DNA fragment containing the gene of interest is identified, it is ligated to an appropriate promoter and secretory signal fragment. Methods of ligation of DNA fragments have been amply described (Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory 1982) and are well within the skill of those of ordinary skill in the art to perform.

After preparation of the v-sis expression constructions, the constructs are inserted into a yeast expression vector.

The replicating plasmid YEp13, containing an origin of replication and a selectable marker, the LEU2 gene, was used for the initial expression constructions. The use of the selectable marker LEU2 in yeast cells deficient in their ability to synthesize leucine allows for the positive selection of those cells containing the LEU2 plasmid by their ability to grow on minus leucine growth media. Although these constructions directed the expression of a product having some mitogenic activity, it is preferable to use an expression vector which is more stably maintained within the host cell in order to produce more mitogenic activity per culture.

Suitable yeast expression vectors in this regard are the plasmids pCPOT and pMPOT, which include the Schizosaccharomyces pombe gene encoding the glycolytic enzyme triose phosphate isomerase (POT1 gene). Inclusion of the POT1 gene ensures the stable maintenance of the plasmid in an appropriate host cell due to its ability to complement the corresponding gene deletion present within this host cell. In addition, the MFα1 promoter was replaced by the Saccaromyces cerevisiae TPI promoter with the intention of further increasing transcription and expression.

After preparation of the DNA construct incorporating the TPI promoter, the alpha factor signal secretory signal sequences, the appropriate segment of the v-sis gene or the human cDNA gene for PDGF, and the TPI terminator in an appropriate vector, the construct is transformed into the yeast host with a TPI deletion. Procedures for transforming yeast are well known in the literature.

The transformed yeast cells may be selected for by growth on conventional complex medium containing glucose when the pCPOT vector is utilized. A conventional medium such as YEPD (20 grams glucose, 20 grams Bacto-peptone, 10

grams yeast extract per liter) may be used. Once selected, transformants containing the v-sis expression constructions are grown to stationary phase on conventional complex media, the cells removed, and the medium concentrated. Noting that authentic human PDGF is a highly cationic and hydrophobic protein (Raines and Ross ibid., Antoniades ibid., Deuel et al., 1981, ibid.), it was expected that the putative yeast product would possess similar characteristics, allowing it to be concentrated on a hydrophobic chromatography matrix such as C8-Sepharose (Pharmacia Fine Chemicals AB, Uppsala, Sweden).

Using a variety of assays, it is demonstrated that growth media from yeast cultures expressing the v-sis derivatives possess biological activities identical to authentic human PDGF.

Expression of biologically active v-sis derivatives in eucaryotic cells other than yeast can be achieved by a person skilled in the art by using the appropriate expression/regulatory signals. Transcriptional promoters capable of directing the expression of v-sis sequences are chosen for their ability to give efficient and/or regulated expression in the particular eucaryotic cell type. Signal sequences capable of directing the v-sis gene product into the cell's secretory pathway are chosen for their function in the appropriate cell type. Other useful regulatory signals, such as transcription termination signals, polyadenylation signals and transcriptional enhancer sequences, are also chosen for their function in the appropriate cell type, the selection of which would be apparent to an individual skilled in the art.

The techniques of cell culture have advanced considerably in the last several years as have the number and varieties of mammalian cells which will grow in culture. Central to these advances is a better understanding of the nutritional requirements (i.e., hormones and growth factors) of cultured cells (Barnes and Sato, Cell 22: 649,

1980). The types of cells able to grow in culture can be crudely classified in two groups: normal and transformed. So-called "normal" cells are generally not immortal in culture, they do not form tumors when injected into animals and they retain a normal diploid karyotype. Normal cells may also retain much of their differentiated character in culture. Within the category of normal cells are those which will only grow for a limited number of generations in culture, termed "cell strains" or "primary cultures." Some normal cell lines, while not meeting all the criteria of transformation, may grow indefinitely in culture. Transformed cells are immortalized for growth in culture, typically have lost their differentiated phenotype, and have acquired karyotypic aberrations. They may also be independent of anchorage for growth and induce tumors when injected into the appropriate host animal. Cells in any of these categories which grow *in vitro* and possess PDGF receptors will be responsive to the PDGF analogs of this invention in culture.

To summarize the examples which follow, EXAMPLE I demonstrates the construction of a v-sis subclone of pSSV-11 in the *E. coli* replicating plasmid pUC13, subsequently designated pVSIS/Pst. EXAMPLE II demonstrates the construction of the plasmid pVSX, which includes the ligation of v-sis to the MFα1 promoter and secretory signal sequence. EXAMPLE III demonstrates the oligonucleotide directed deletion mutagenesis of the first 195 base pairs of the v-sis gene using a technique which employs single stranded bacteriophage M13, in order to eliminate the first sixty-six amino acids of the v-sis gene product, p28sis, which are not homologous to the B chain of PDGF. A resulting phage with the correct deletion was designated m11vs2α. EXAMPLE IV demonstrates the construction of the plasmid pVSB. EXAMPLE V demonstrates the incorporation of the v-sis related constructions described in Examples II and III into the yeast replicating vector YEp13 and addition of

yeast TPI terminator sequences. Subsequently, VS2α sequences were inserted into the plasmid pCPOT, which ensures the stable maintenance of the plasmid in the host cell. This plasmid was designated pl17-2. EXAMPLE VI demonstrates the transformation of yeast host cells with the plasmids YEpVSα, YEpVS2α, pl17-2 and control plasmids p270 and pCPOT, and subsequent transcriptional analysis. EXAMPLE VII demonstrates the concentration of the spent yeast growth media from cultures containing the v-sis expressing transformants and their subsequent analysis for PDGF-like material by the ELISA, radioreceptor and mitogenesis assays. Clear evidence is presented that these yeast media containing the v-sis related gene products described herein possess biological activities identical to authentic human PDGF.

The following examples are offered by way of illustration, and not by way of limitation.

EXAMPLES

Unless otherwise indicated, standard molecular biological methods were used. Restriction endonucleases and other DNA modification enzymes (i.e., T_4 polynucleotide kinase, calf alkaline phosphatase, Klenow DNA polymerase) were obtained from Bethesda Research Laboratories, New England Biolabs, Boehringer-Mannhiem or Collaborative Research and were used as the manufacturer suggested unless indicated otherwise. M13 phage and pUC plasmid vectors and appropriate host strains were obtained from Bethesda Research Laboratories. *E. coli* cultures were transformed by the calcium chloride method of Dagert and Ehrlich (*Gene* 6: 23, 1979). Yeast cultures were transformed as described by Beggs (*Nature* 275: 104, 1976). Plasmid and M13 replicative form (RF) DNA were prepared from *E. coli* transformants by the method of Birnboim and Daly (*Nucleic Acids Research* 7: 1513, 1979). Single stranded M13 phage DNA was pre-

pared as described by S. Anderson (Nucleic Acids Research 13: 3015, 1981). DNA fragments were extracted from agarose gels by the method of J. Langridge et al. (Analyt. Biochem. 103: 264, 1980). DNA sequencing was performed by the dideoxy method on M13 templates (Messing, Meth. in Enzymology 101: 20, 1983).

EXAMPLE I

Subcloning of V-SIS from pSSV-11

The SSV retroviral genome was cloned from SSV-11 nonproductively infected normal rat kidney (NRK) cells which had SSV integrated into their genome (Devare et al., 1982, ibid.). The SSV DNA was isolated as a 5.8 kilobase (kb) Eco RI fragment and subsequently inserted into the plasmid pBR322, resulting in the clone pSSV-11. This clone was obtained from S. Aaronson (National Institutes of Health, Bethesda, MD).

Figure 1A is a schematic restriction map of the 5.8 kilobase proviral genome of SSV. Only the restriction sites relevant to the present invention are indicated. The open box designates the p28sis coding portion of the v-sis gene.

Figure 1B depicts the nucleotide sequence of the v-sis gene and some flanking SSV sequences. The v-sis gene is inserted 19 nucleotides 3' of the putative ATG initiation codon of the envelope (env) gene of SSV (Devare et al., 1982, ibid.). It is believed that transcription and translation of v-sis sequences are directed by SSV sequences resulting in an env-sis fusion protein. The nucleotide sequence shown in Figure 1B is corrected from that published by Devare et al. in 1982 (ibid.). The corrections include those made by Devare et al. in 1983 (ibid.) and by the inventors herein. The original numbering scheme of Devare et al. (1982, ibid.) is retained here

for ease of reference. The numbers assigned to the restriction sites in Figure 1A are from Figure 1B.

A subclone of pSSV-11 (Figure 2) containing a portion of the v-sis gene was constructed in the E. coli replicating plasmid pUC13 (Vieira and Messing, Gene, 19: 259, 1982; and Messing, Meth. in Enzymology 101: 20, 1983). Five micrograms (ug) of pSSV-11 was digested with the restriction endonuclease Pst I and the 1.2 kb fragment containing sequences numbered 454-1679 (Figure 1) was purified by agarose gel electrophoresis (0.9%) and extracted from the gel with cetyltrimethylammonium bromide (CTAB) plus butanol (Langridge et al., ibid.). Two ug of pUC13 was also digested with Pst I, phenol/chloroform ($CHCl_3$) extracted and ethanol (EtOH) precipitated. Forty ng of the 1.2 kb v-sis fragment and 50 ng of Pst I cut pUC13 were ligated overnight at room temperature with 40 units (u) of T_4 DNA ligase. The ligation mixture was used to transform E. coli K-12 strain JM83 (Messing, Recombinant DNA Technical Bulletin, NIH Publication No. 79-009, 2, No. 2, 43-48, 1979) in the presence of 5-bromo,4-chloro, 3-indolyl-B-D-galactoside (X-gal) and isopropyl B-D-thiogalactoside (IPTG). Plasmid DNA prepared from ampicillin resistant white colonies was digested with Pst I to verify the presence of the insert and the resulting plasmid was designated pVSIS/Pst.

EXAMPLE II

Construction of the Plasmid pVSα

A. Preparation of V-SIS for Fusion to MFα1.

Six hundred ug of plasmid pSSV-11 (Figure 2) was digested with restriction endonucleases Bam HI and Pvu II in 200 microliters (ul) of 50 mM NaCl, 10 mM $MgCl_2$, 10 mM Tris pH 7.5 (medium salt buffer), and 100 ug/ml bovine

serum albumin (BSA), overnight at 37°C. The digestion products were electrophoresed through a 1.1% agarose gel and the 1100 base pair (bp) Bam HI-Pvu II fragment (Figure 2) cut out, extracted and EtOH precipitated. The DNA pellet was dissolved in 75 ul Hph I buffer to which was added 20 ul of 1 mg/ml BSA and 5 ul Hph I. After overnight digestion at 37°C the mixture was electrophoresed through a 1.25% agarose gel and the 396 bp Hph I-Pvu II fragment isolated from the gel and EtOH precipitated. The DNA pellet was dissolved in 30 ul of Klenow buffer (6mM Tris pH 7.5, 6 mM $MgCl_2$, 60 mM NaCl) and the 3' overhanging nucleotide at the Hph I cleavage site removed by treatment with 5 u of Klenow polymerase for 5 minutes at 37°C. One ul of a mixture containing all four deoxyribonucleotides each at 1 mM was added and the reaction mixture incubated an additional 10 minutes. After phenol/$CHCl_3$/ether (Et_2O) extraction and EtOH precipitation, the DNA pellet was dissolved in 30 ul of medium salt buffer and digested with 5 u of Bgl II for three hours at 37°C. The DNA was electrophoresed through a 1.25% agarose gel and the 269 bp Hph I - Bgl II fragment extracted and EtOH precipitated. The Hph I cleavage terminus of this Klenow blunted fragment begins with the tri-nucleotide sequence

5'ATG.....(Figure 2)
3'TAC.....

B. MFα1 Promoter and Secretory Leader Fragment.

Plasmid pl92 (Figure 3) comprises a portion of the gene for the yeast mating pheromone α-factor (MFα1 gene) cloned in the bacterial plasmid pUC13 (Vieira and Messing, ibid.; and Messing, Meth. in Enzymology 101: 20, 1983). Cloning of the MF 1 gene from a genomic library has been described by Kurjan and Herskowitz (ibid.). The gene was isolated in this laboratory in a similar manner, using as starting material a yeast genomic library of partial Sau

3A fragments cloned into the Bam HI site of Yepl3 (Nasmyth and Tatchell, Cell 19: 753, 1980). From this library, a plasmid was isolated which expressed α-factor in a diploid strain of yeast homozygous for the mat 2-34 mutuation (Manney et al., J. Cell Biol 96: 1592, 1983). The clone contained an insert overlapping with the MFα1 gene characterized by Kurjan and Herskowitz (ibid). This plasmid, known as p2A2 (Figure 3), was cut with Eco RI and the 1700 bp fragment comprising the MFα1 gene was purified. This fragment was then subcloned into the Eco RI site of pUC13 to produce the plasmid pl92.

Fifteen ug of plasmid pl92 was digested in 30 ul of medium salt buffer with 20 units of Hind III overnight at 37°C. The reaction mixture was diluted to 60 ul with Klenow buffer and the four deoxyribonucleotides added to a final concentration of 50 uM each. Ten units of Klenow polymerase were added to the ice-cold mixture and incubation allowed to proceed 12 minutes at 15°C. Following phenol/$ChCl_3$/Et_2O extraction, the aqueous phase was concentrated by lyophilization to a volume of 10 ul and digested with 20 units of Eco RI for 70 minutes at 37°C. The products were electrophoresed through a 0.9% agarose gel and the 1.2 kb Eco RI-Hind III (blunted) MFα1 fragment extracted and EtOH precipitated. This DNA fragment contains the transciptional promoter and secretory signal sequences of MFα1.

C. Preparation of v-sis 3' Sequences and Cloning Vector pUC12; Fragment Ligation.

Twenty ug of plasmid pVSIS/Pst was digested with Bgl II and Xba I in 40 ul of medium salt buffer. Subsequent electrophoresis through 1% agarose, extraction of the DNA and EtOH precipitation provided the purified v-sis 756 bp Bgl II-Xba I fragment (Figure 2). E. coli replicating

plasmid pUC12 (5 ug) was digested with Eco RI and Xba I and gel purified as above (Figure 2).

Referring to Figure 2, equimolar amounts of the four DNA fragments described above, adjusted to 10 ng of the 296 bp Hph I-Bgl II v-sis fragment, were mixed in 15 ul of ligase buffer (6 mM Tris pH 7.6, 6.6mM $MgCl_2$, 0.4 mM ATP, 2 mM spermidine, 20 mM DTT, and 100 ug/ml BSA) and ligated with 40 units of T_4 DNA ligase overnight at 14°C. The reaction mixture was brought to room temperature, an additional 150 units of T_4 ligase added, and incubated 10 more hours. Seven ul of the ligation mix was used to transform E. coli K-12 RR1 (ATCC #31343; Bolivar, E. et al., Gene 2: 95, 1977), and ampicillin resistant transformants selected. Plasmid DNA was prepared from 12 such bacterial colonies and digested with Xba I. Two clones gave a ~2.2 kb band predicted by the proper fragment alignment (Figure 2). Further analysis of these by Bgl II-Xba I restriction mapping gave expected bands of approximately 1.5 kb from the MFα1/v-sis fusion and 760bp for the Bgl II-Xba I v-sis fragment. DNA sequence analysis verified the desired nucleotide sequence at the MFα1/v-sis junction. The resultant plasmid was designated pVSα.

EXAMPLE III

Oligonucleotide Directed Deletion Mutagenesis of 66 Amino Terminal v-sis codons

Homology between the v-sis protein $p28^{sis}$, and PDGF begins at amino acid 67 of $p28^{sis}$, a serine residue corresponding to the NH_2 terminal residue of the PDGF B chain (Johnsson, ibid.)

Proteolytic processing of the MFα1 primary translation product occurs at the Lys-Arg cleavage signal 85 amino acids from the initiator methionine (Kurjan and

Herskowitz, ibid.). A v-sis derivative was constructed in which the first 66 codons of p28sis were removed such that serine residue 67 of v-sis immediately follows the MFα1 Lys-Arg processing signal.

Referring to Figure 4, approximately 40 ng of the gel purified 2.2 kb Xba I fragment of pVSα was ligated with 120 ng of Xba I digested, alkaline phosphatase treated M13mp11 DNA (Messing, Meth. in Enzymology, ibid.). The ligation mixture was used to transform E. coli K-12 strain JM101 (ATCC 33876) in the presence of X-gal and IPTG. Isolated white plaques were picked and used to infect 3 ml cultures of log phase growth JM101 cells. Replicative Form (RF) DNA was prepared and clones identified which carried the insert fragment in the same orientation as the positive (+) strand form of the single stranded mature phage. Single-stranded phage DNA was prepared from one such clone and designated m11VSα.

To precisely remove codons 1-66 of v-sis, oligonucleotide directed mutagenesis was performed essentially according to the two primer method of Zoller (Zoller, et al., Manual for Advanced Techniques in Molecular Cloning Course, Cold Spring Harbor Laboratory, 1983). Oligonucleotide ZC 130 3' AGAAACCTATTTTCCTCGGACCCA 5' was synthesized on an Applied Biosystems 380-A DNA synthesizer. Fifty pmoles of ZC 130 were kinased in 10 ul of kinase buffer (BRL) with 4 units of T_4 polynucleotide kinase for 45 minutes at 37°C. The enzyme was inactivated by heating at 65°C for 10 minutes.

One-half pmole of m11VSα was annealed with 1 pmole of kinased ZC 130 and 1.5 pmoles of universal sequencing primer (BRL) using conditions described (Zoller, ibid.), except that the annealing mixture was first heated to 65°C for 10 minutes, shifted to 37°C for 10 minutes, and then quickly chilled on ice. The annealed mixture was then treated with Klenow polymerase as described by Zoller (ibid.) to create circular duplex DNA. Portions of the

elongation mixture were used to transform E. coli K12 JM 101 cells. The resulting phage plaques were screened for the proper deletion by transfer onto nitrocellulose filters and subsequent hybridization with ^{32}P phosphorylated ZC 130 at 65°C. Correctly juxtaposed sequences formed stable duplexes with the radioactive probe at the stringent hybridization temperature employed. Approximately 1% of the transformants screened gave positive signals by autoradiography. Ten clones were plaque-purified and RF DNA was prepared for restriction enzyme analysis. Five isolates showed the expected decrease in size of 195 bp to the 1450 bp Hind III-Bgl II fragment (Figure 4). DNA sequence analysis of two isolates confirmed the correct fusion junction had been made, thus maintaining the proper translational reading frame. One of these phage was designated m11VS2α.

EXAMPLE IV

Construction of the Plasmid pVSB

Because the product encoded by pVS2α is larger than authentic human PDGF B chain and because a smaller product might result in higher expression levels in a transformed yeast host cell, a vector was constructed comprising the v-sis sequence of pVS2α truncated at the 3' end. The polypeptide encoded by this sequence comprises amino acids 67 to 175 of $p28^{sis}$ and is homologous to the B chain of PDGF.

An expression vector containing this "B chain" sequence was constructed by combining elements of the pVS2α expression unit with a partial v-sis gene and a synthetic double-stranded DNA fragment encoding amino acids 158 to 175 of $p28^{sis}$. This synthetic fragment was designed to substitute preferred yeast codons for many of the 13 v-sis codons it replaces, and to supply a stop codon at the end

of the coding sequence. The construction of this vector is illustrated in Figures 8 and 9.

Plasmid YEpVS2 was digested with Pst I and Bam HI and the 1.8 kb fragment comprising the partial MF 1, v-sis, and TPI terminator sequences was purified by agarose gel electrophoresis. Plasmid pIC19R (obtainable from Dr. J. Lawrence Marsh, University of California, Irvine), comprising the polylinker shown in Chart 1 inserted into the Hind III site of pUC19 (Norrander et al., Gene 26: 101-106, 1983), was digested with Pst I and Bam HI, and the vector fragment was gel purified and joined to the 1.8 kb fragment from pVS2∝ to produce plasmid pVS2∝T.

CHART 1

GAATTCATCGATATCTAGATCTCGAGCTCGCGAAAGCTT
Eco Rl Eco RV Bgl II Sac I Hind III
Cla I Xba I Xho I Nru I

Plasmid pM220 was digested with Bgl II and Pst I, and the ca. 1 kb fragment comprising the TPI promoter and the 5' portion of the MF∝1 sequence was isolated and cloned in Bgl II + Pst I digested pIC19R. The resultant plasmid was digested with Cla I and Pst I, and the TPI promoter - MF∝1 fragment was gel purified. Plasmid pVS2∝T was then cut with Cla I and Pst I and joined to the TPI promoter - MF∝1 fragment. The correct construct was identified by the presence of a 2.6 kb Cla I - Bam HI fragment and was designated pTVS2∝T.

Ten ug of plasmid pVS∝ was digested with Xma I and Sph I to completion. The resulting ca. 4.9 kb vector fragment, which also comprises most of the v-sis sequence, was purified by agarose gel electrophoresis, extraction of the DNA and EtOH precipitation.

In order to supply a new 3' terminus for the v-sis sequence, a double-stranded DNA fragment was

constructed from oligonucleotides synthesized on an Applied Biosystems Model 380-A DNA synthesizer. 0.7 pmole of oligonucleotide ZC299 (Table 1) was heated with an equimolar amount of oligonucleotide ZC300 in a volume of 10 ul containing 40 mM NaCl for 5 minutes at 65°C.

TABLE 1

ZC299: 5'TAAG TGT GAA ATC GTT GCC GCG GCT AGA GCT GTT ACC TAA TCT AGA$^{3'}$

ZC300: 3'GTACA TTC ACA CTT TAG CAA CGG CGC CGA TCT CGA CAA TGG ATT AGA TCT GGCC$^{5'}$

The mixture was then incubated at 37°C for 5 minutes and allowed to cool to room temperature. 0.2 pmole of the purified 4.9 kb vector fragment was added, the mixture ligated for 18 hours at 12°C and used to transform E. coli HB101 (ATCC 33694) to Ampicillin resistance. DNA was prepared from Ampicillin-resistant colonies and digested with Bgl II and Xba I. After electrophoresis through agarose, the desired clone (known as pVSXB) was identified by loss of a ca. 750 bp Bgl II--Xba I fragment and appearance of two smaller fragments of approximately 500 and 260 bp.

Approximately 8 ug of plasmid pTVS2 T was digested to completion with Xba I in a volume of 10 ul. The volume was increased to 40 ul with Bgl II buffer, and 6 units of Bgl II were added and the mixture was incubated at 37°C. Ten ul aliquots were removed to a stop buffer containing 50 mM EDTA at 15 and 30 minutes, and the remaining 20 ul stopped at 45 minutes. The resulting mixtures were separated by electrophoresis through 0.7% agarose. The ca. 4.6 kb Bgl II--Xba I vector fragment was cut out, extracted from the gel, and EtOH precipitated.

Plasmid pVSαB was digested with Bgl II and Xba I, and the ca. 260 bp fragment containing the synthetic 3' terminus and stop codon was isolated by electrophoresis through agarose, subsequent extraction from the gel, and EtOH precipitation.

The 4.6 kb Bgl II-Xba I vector fragment from pTVS2αT and the 260 bp Bgl II--Xba I fragment from pVSαB were ligated in the presence of T4 DNA ligase for 7 hours at room temperature. The reaction mixture was used to transform E. coli HB101 to Ampicillin resistance. DNA was prepared from transformants and the presence of the desired insert was confirmed by screening for a 550 bp Pst I--Xba I band on an agarose gel. A plasmid having the correct configuration was designated pVSB.

EXAMPLE V

Yeast Expression Vectors

A. Construction of Plasmids YEpVSα and YEpVS2α

Yeast Replicating Vector YEp13 (Broach, et al., Gene 8: 121, 1979) was used as an expression vehicle for v-sis derived constructions described in Examples II and III. YEp13 is a multicopy extrachromosomal plasmid containing a 2 micron replication origin and the yeast LEU2 gene. This allows for selection of the plasmid in yeast strains possessing a defective chromosomal LEU2 gene when grown on synthetic medium lacking leucine. Addition of yeast terminator sequences to foreign genes expressed in yeast ensures efficient transcription termination and polyadenylation of mRNA. The v-sis expression units VSα and VS2α were placed adjacent to the TPI terminator fragment which was preivously cloned into YEp13 (below).

Plasmid p270 (see Figure 5) contains the transcription terminator region of the yeast triose phosphate isomerase (TPI) gene. It was constructed in the

following manner. The yeast TPI terminator fragment was obtained from plasmid pFG1 (Albert and Kawasaki, ibid.). It encompasses the region from the penultimate amino acid codon of the TPI gene to the Eco RI site approximately 700 base pairs downstream. A Bam HI site was substituted for this unique Eco RI site of pFG1 by first cutting the plasmid with Eco RI, then blunting the ends with DNA polymerase I (Klenow fragment), adding synthetic Bam HI linkers (CGGATCCA), and re-ligating to produce plasmid p136. The TPI terminator was then excised from p136 as a Xba I-Bam HI fragment. This fragment was ligated into YEp13 (Broach, et al., ibid.) which had bene linearized with Xba I and Bam HI. The resulting plasmid is known as p213. The Hind III site was then removed from the TPI terminator region of p213 by digesting the plasmid with Hind III, blunting the resultant termini with DNA polymerase I (Klenow fragment), and recircularizing the linear molecule using T_4 DNA ligase. The resulting plasmid is p270.

Alternatively, p270 may be constructed by digesting plasmid pM220 (see below) with Xba I and Bam HI, purifying the TPI terminator fragment (~700bp) and inserting this fragment into XbaI and Bam HI digested YEp13.

Referring to Figure 6, plasmid p270 DNA was digested with Xba I and treated with calf alkaline phosphatase to prevent religation of the cohesive vector ends. V-sis expression units VS$^{\alpha}$ and VS2$^{\alpha}$ were prepared by Xba I digestion and agarose gel purification of pVS$^{\alpha}$ and m11vs2$^{\alpha}$, respectively. Each of the isolated fragments was ligated with an approximately equimolar amount of phosphatased p270 vector in the presence of 40 units of T_4 DNA ligase and the ligation mixtures transformed into E. coli K-12 RR1. Plasmid DNA was prepared from ampicillin-resistant colonies and restriction enzyme analysis performed in order to identify clones which possessed the TPI terminator adjacent to 3' v-sis sequences. Presence of 3.3 kb or 3.1 kb Bgl II

fragments after gel electrophoresis indicated the correct orientation of YEpVSα and YEpVS2α, respectively.

B. Insertion of VS2α Expression unit into pCPOT.

In order to achieve maximal protein production from a yeast culture, it is desirable to use expression vehicles which are very stably maintained in the host cell. Plasmid pCPOT is such a preferred expression vehicle.

E. coli HB101 transformed with pCPOT has been deposited with American Type Culture Collection under accession number 39685. Plasmid pCPOT comprises the 2 micron circle genome (Hartley and Donelson, *Nature* 286: 860, 1980), *E. coli* plasmid pBR322 replication and selection sequences, and the *Schizosaccharomyces pombe* DNA sequences encoding the glycolytic enzyme Triose Phosphate Isomerase (*POT1*). Presence of the *POT1* gene in pCPOT ensures stable maintenance of the plasmid in the appropriate host background during growth on nonselective medium utilizing glucose as a carbon source.

The *S. cerevisiae* TPI promoter was used to control expression of VS2α sequences in pCPOT. Plasmid pM220 contains the TPI promoter fused to the MFα1 signal sequence. *E. coli* RRI transformed with pM220 has been deposited with American Type Culture Collection under accession number 39853.

Referring to Figure 7, plasmid pM220 was digested with Bgl II and Bam HI, electrophoresed through a 0.9% agarose gel, and the 2.2 kb TPI promoter, MFα1 gene fragment extracted. The purified fragment was digested with Pst I and the resulting 1 kb Bgl II-Pst I fragment agarose gel-purified as above. Plasmid YEpVS2α was digested with Pst I and Bam HI, and the 1.8 kb MFα1/v-sis/TPI terminator fusion fragment gel-isolated. Plasmid pCPOT was digested with Bam HI, treated with calf alkaline phosphatase, phenol/$CHCl_3$ extracted, then purified by electrophoresis

through agarose, extracted from the gel and EtOH precipitated.

Approximately equimolar amounts of the three isolated fragments described above (Figure 7) were ligated overnight at 12°C and the ligation mixture used to transform *E. coli* K-12 strain DH1 (Hanahan, D. and Meselson, M., *J. Mol. Biol.* 166: 577, 1983) to ampicillin resistance. Plasmid DNA was prepared from transformants and restriction digest analysis used to ascertain the orientation of the insert fragments. Presence of the ~1500 bp Bam HI-Sal I fragment indicates that the Bam HI cohesive end of the TPI terminator fragment is oriented as shown in Figure 7. The opposite orientation would create a Bam HI/Bgl II fusion, not cleavable by Bam HI, and hence would not yield this fragment. The 800 bp Sph I fragment indicated that TPI promoter and v-sis fragments were properly fused at the Pst I site (Figure 7). This plasmid was designated pl17-2.

For expression of the v-sis derivatives in yeast, a stable expression vector comprising the REP1, REP2, REP3 and ori sequences from yeast 2 micron DNA and the *Schizosaccharomyces pombe* triose phosphate isomerase (POT1) gene was constructed. The POT1 gene provides for plasmid maintenance in a transformed yeast host grown in complex media if such host is defective for triose phosphate isomerase.

The POT1 gene was obtained from the plasmid pFATPOT. *S. cerevisiae* strain E18 transformed with pFATPOT has been deposited with ATCC under accession number 20699. The plasmid may be purified from the host cells by conventional techniques. The POT1 sequence was removed from pFATPOT by digestion of the plasmid with Sal I and Bam HI. This 1600 bp fragment was then ligated to pIC19R, which had first been linearized by digestion with Sal I and Bam HI. The Bam HI, Pst I and Sal I sites in the resultant plasmid were destroyed in two steps to produce plasmid pICPOT*. The Pst I and Sal I sites were removed by cutting with Pst I and Sal I; the ends were blunted by digesting

the Pst I 3' overhang with DNA polymerase I (Klenow fragment) and filling in the Sal I 5' overhang with Klenow fragment. The blunt ends were then ligated. The Bam HI site was then removed by cutting the plasmid with Bam HI, filling in the ends with DNA polymerase I (Klenow fragment) and religating the blunt ends.

The 2u sequences were obtained from the plasmids YEp13 (Broach et al., Gene 8: 121-133, 1979) and C1/1. C1/1 was constructed from pJDB248 (Beggs, Nature 275: 104-109, 1978) by removal of the pMB9 sequences by partial digestion with Eco RI and replacement by Eco RI-cut pBR322. The REP3 and ori sequences were removed from YEp13 by digestion with Pst I and Xba I and gel purification. REP2 was obtained from C1/1 by digestion with Xba I and Sph I and gel purification. The two fragments were then joined to pUC18 (Norrander et al., Gene 26: 101-106, 1983) which had been linearized with Pst I and Sph I to produce plasmid pUCREP2,3. REP1 was obtained from C1/1 by digestion with Eco RI and Xba I and gel purification of the 1704 bp fragment. The Eco RI--Xba I fragment was cloned into pUC13 which had been linearized with Eco RI and Xba I. The resultant plasmid was designated pUC13 + REP1. The pUC13 + REP1 plasmid was cut with Hind II and ligated in the presence of Eco RI linkers (obtained from Bethesda Research Laboratories). The REP1 gene was then removed as an Eco RI fragment of approximately 1720 bp. This Eco RI fragment was cloned into pIC7 (comprising the polylinker sequence shown in Figure B inserted into the Hind III site of pUC8), which had been linearized with Eco RI and Xba I. The resultant plasmid was designated pICREP1#9.

To construct the final expression vector pMPOT2, pICPOT* was linearized by a partial Hind III digestion and complete Sst I digestion. Plasmid pUCREP2,3 was cut with Hind III and Sst I, and the fragment comprising REP2, REP3 and ori sequences was gel purified and joined to the linearized pICPOT*. The resultant plasmid, comprising

REP2, REP3, ori, POT1 and amp^r sequences, was designated pMPOT1. REP1 was then removed from pICREP1 as a Bgl II--Nar I fragment and was ligated to pMPOT1, which had been cleaved with Bgl II and Nar I. The product of this ligation was designated pMPOT2 (deposited with ATCC, accession number not yet assigned). Plasmid pMPOT2 was digested with Cla I and Bam HI, and the vector fragment was purified as above.

C. Insertion of v-sis Expression Units in pMPOT.

1. Insertion of VSα expression unit into pMPOT2.

Approximately 10 ug of plasmid pVSα was digested with Bst EII to completion in a volume of 20 ul. Five units of Pst I were added, the mixture was incubated 30 minutes and the reaction stopped by the addition of EDTA. The quenched reaction mixture was immediately electrophoresed through a 1% agarose gel, and the ca. 800 bp partial Pst I--Bst EII band (comprising most of the MFα1 prepro sequence and the 5' portion of v-sis) was cut out, extracted from the gel, and EtOH precipitated.

Plasmid pTVS2αT was digested to completion with Pst I and Bst EII and purified by agarose gel electrophoresis. The resulting ca. 4.8 kb vector fragment and the 800 bp Pst I--Bst EII fragment were ligated in the presence of T_4 DNA ligase for 6 hours at room temperature, and the ligation mixture was used to transform E. coli HB101 to ampicillin resistance. A plasmid was identified which contained a ca. 1450 bp Bgl II fragment, which indicated the presence of the insert. It was designated pTVSα.

Plasmid pTVSα was digested to completion with Cla I and Bam HI, and the ca. 2.9 kb fragment containing VSα sequences was isolated by electrophoresis through agarose, extraction from the gel, and EtOH precipitation. The ca. 2.9 kb Cla I--Bam HI VSα fragment was ligated with Cla I

and Bam HI digested pMPOT2 as described for pVS2αm (below).

A plasmid containing a 2.9 kb Cla I--Bam HI insert was identified and designated pVSαm.

2. Insertion of VS2α expression unit into MPOT2.

Plasmid pTVS2αT was digested to completion with Cla I and Bam HI in Bam HI buffer. The buffer was adjusted to high salt (Maniatis et al, ibid.) and the DNA was digested to completion wit Pvu I, which cuts the vector sequences twice and permits resolution of the ca. 2.7 kb Cla I--Bam HI fragment containing the VS2 sequences on an agarose gel. This fragment was electrophoresed through 0.9% agarose, extracted, and EtOH precipitated. The fragment was then ligated with Cla I--Bam HI digested pMPOT2 in the presence of T4 DNA ligase for 20 hours at 13°C. The ligated DNA was used to transform E. coli HB 101 to ampicillin resistance, and plasmid DNA was prepared from the resulting colonies. A plasmid was identified which contained the 2.7 kb Cla I--Bam HI VS2 fragment and was designated pVS2αm.

3. Insertion of VSB expression unit into pMPOT2.

Plasmid pVSB was digested with Cla I and Bam HI, and the 2.2 kb fragment containing the "B chain" expression unit purified by agarose gel electrophoresis and EtOH precipitation. The fragments were ligated overnight at room temperature in the presence of T4 DNA ligase and the reaction mixture used to transform E. coli HB101 to ampicillin resistance. DNA was prepared from transformants and the presence of the insert verified by digestion with Cla I and Bam HI and agarose gel electrophoresis. The resulting expression vector was designated pVSBm.

EXAMPLE VI

Yeast Transformation; and
Analysis of v-sis Transcription

S. cerevisiae strain E8-11c (MATα leu2-3, 112 pep 4-3; a haploid segregant of the cross E2-7B [ATCC 20689[x GK 100 [ATCC 20669]) was transformed with plasmids YEpVSα, YEpVS2α, p270, p117-2 and pCPOT. Transformants were selected and maintained in synthetic medium lacking leucine.

S. cerevisiae strain E11-3c (ATCC Accession #20727) (MATα pep4-3 tpi1) was transformed with plasmids pCPOT and p117-2. Transformants were selected and maintained in YEPD.

Referring to Figure 8, presence of v-sis related mRNA transcripts was confirmed by electrophoretic and subsequent hybridization analysis of total RNA. Total RNA from the above described transformants in strain E8-11c was prepared by guanidinium thiocyanate extraction as described by Maniatis et al. (ibid.) with the following modifications: 100ml cultures were grown to a density of $1x10^8$ cells/ml. The cells were pelleted by centrifugation and washed three times with H_2O, 2 mls of guanidinium lysis solution was added, followed by 0.5mm glass beads to just below the meniscus. The tubes were vortexed three times for 1 minute with cooling on ice between bursts. The solution was pipetted off and the RNA isolated by centrifugation through $CsCl_2$ as described (Maniatis et al., ibid.). Fifteen ug of RNA from plasmid transformants p270, YEpVSα, YEpVS2α;, pCPOT and p117-2 was glyoxylated, electrophoresed through a 0.9% agarose gel and transferred to nitrocellulose as described by Thomas (PNAS 77: 5201, 1980). The purified Pst I v-sis fragment from pVSIS/Pst was nick translated and hybridized to the filter bound RNA, and the hybridizing species detected by autoradiography (Figure 10). Tran-

script bands of 1900 bp from YEpVSα, ~1650 bp from YEpVS2α, and ~1700 bp from p117-2 confirmed the transcription of the v-sis fusion constructs and the use of the transcription start and stop signals in the constructions. No v-sis related transcripts were detected in negative controls p270 and pCPOT.

Plasmids pVSαm, pVS2αm, pVSBm, and pMPOT2 were used to transform S. cerevisiae strain E18. Strain E18 is a diploid produced by crossing strains E11-3c (ATCC No. 20727) and δ tpi 29. δ tpi 29 is produced by disrupting the triose phosphate isomerase gene of strain E2-76 (ATCC No. 20689), essentially as described by Rothstein (Methods Enzymol 101: 202-210, 1983).

EXAMPLE VII

Analysis of sis-related Products Expressed by Yeast; and Biological Activity Assays

A. Concentration of Yeast Culture Medium.

Transformants carrying YEp13 and pCPOT derived v-sis constructions were grown in the appropriate media at 30°C (1.2 liter cultures) to stationary phase on a rotary air shaker with agitation at 220 rpm. Cultures were harvested, the cells removed by centrifugation, and the medium concentrated on a C-8 Sepharose (Pharmacia Fine Chemicals AB, Uppsala, Sweden) column which binds molecules of a hydrophobic nature. Authentic human PDGF is a highly cationic and hydrophobic protein (Heldin et al., PNAS 76: 3722, 1979; Raines and Ross, ibid.). The sis-related putative yeast product was expected to possess similar characteristics. The sis products expected hydrophobic character was exploited to concentrate it from the yeast media into which it was expected to be secreted. Molecules bound to the C-8 column are eluted from the matrix with suitable hydrophobic solvents.

Spent growth media from the transformed yeast cultures was adjusted to 5% EtOH and passed through an 8 ml C-8 Sepharose column at a flow rate of 2-3 ml per minute. The column was then washed with 100mls of 5% EtOH in 20 mM ammonium bicarbonate (NH_4HCO_3). The bound material was eluted with 20% propanol in 20mM NH_4HCO_3 and the eluate collected in 1-2 ml fractions. Fractions were assayed for protein content by light absorption at 280 nm, (A_{280} of 1.4=1.0 mg protein/ml) or by the method of Lowry et al. (J. Biol. Chem. 193: 265, 1951). The concentrated fractions were combined, lyophilized, and then resuspended in 500-700 ul of PBS (phosphate buffered saline, pH 7.4).

Transformant pli7-2 in strain El1-3c grown under POT1 selection (with glucose as carbon source) was expected to produce significantly higher levels of PDGF-like material in the media and thus was analyzed after dialysis of the media against PBS without concentration.

Media samples from the transformants pVSαm, pVS2αm, pVSBm and pMPOT2 were concentrated by adsorption to CM-sephadex and elution with 1M NaCl in 1M acetic acid, pH 4.5. The concentrated media were dialyzed against 0.1 M acetic acid, pH 7 and the amount of PDGF-like material in the concentrates was determined by ELISA.

B. Detection of PDGF-like Material By Enzyme-Linked Immunosorbent Assay (ELISA)

The expression of PDGF-like molecules by the yeast transformants was examined by ELISA and quantitated by comparison to a standard curve developed with purified human PDGF (Raines and Ross, ibid.). A typical standard curve was prepared as follows:

Purified human PDGF, 2.5 ng/ml in PBS, was incubated overnight with Immulon II (Dynatech Laboratories, Inc.) 96 well microtiter plates (100 ul/well) at 4°C. This coating solution was removed and 100 ul/well of 0.1% rabbit albumin

in PBS was added and the plates incubated for 1 hour at 37°C. Samples of purified PDGF (0.1-40ng/ml) were separately incubated with goat anti-PDGF IgG (5 ug/ml) in PBS containing 0.05% Tween 20 and 1 mg/ml rabbit albumin (RSA). The microtiter plates were washed 5 times with 0.9% NaCl, .05% Tween 20, drained, and 100 ul of each test solution was added to the microtiter wells and incubated 2 hours at 37°C. The plates were washed as before, and peroxidase-conjugated swine anti-goat IgG (Tago, Inc.) diluted 1:1000 in PBS containing 0.05% Tween 20 and 1 mg/ml RSA was added for 2 hours at 37°C. The plates were washed as before and freshly prepared .04% o-phenylene diamine containing .012% hydrogen peroxide (H_2O_2) (100 ul/well) was added for 50 minutes at room temperature and the reaction stopped at 50 minutes by the addition of 4N H_2SO_4 (50 ul/well). Absorbance at 492 nm was determined using a Dynatech plate scanner. Each test point was measured in triplicate and plotted as the mean ± standard error. C-8 eluates of yeast culture media and unconcentrated media samples were diluted in PBS, assayed as described and compared to the PDGF standard curve. Table 2 is a summary of assay results for a representative series of experiments. Figure 11 depicts an ELISA of a range of C-8 eluate sample volumes measured, generating a dose-response curve which is compared to a standard curve from purified PDGF.

Raw ELISA data for the MPOT constructions is not shown, but has been incorporated into the radioreceptor and mitogenesis assay data as shown in Figures 13 and 14.

C. Radioreceptor Assay (RRA) for PDGF.

The radioreceptor assay for PDGF (Bowen-Pope and Ross, J. Biol. Chem. 257: 5161, 1982) is a specific and sensitive (.2-2 ng/ml PDGF) method for detecting biologically active PDGF-like material in yeast. In this assay, PDGF-like material is tested for its ability to compete

with purified, radio-labeled ^{125}I PDGF for binding sites on cell surface PDGF receptors. Results are interpreted by comparison to a standard curve generated with purified, unlabeled PDGF. Comparison of results obtained with other assay methods (e.g., ELISA) provides an indication of the strength of the receptor/ ligand interaction in addition to quantitation of the material bound. The assay is conducted as follows: Subconfluent monolayers of diploid human fibroblasts are prepared by plating 1.5×10^4 cells per $2cm^2$ culture well in Costar 24 well cluster trays in Dulbeccos Modified Eagles Medium (DMEM) supplemented with 1% human plasma-derived serum (PDS). Cultures are set on an ice tray and rinsed once with ice-cold binding rinse (Ham's medium F-12 buffered at pH 7.4 with 25mM HEPES and supplemented with 0.25% BSA). One ml/well of test substance in binding medium is added and the cultures incubated in a refrigerated room on an oscillating platform for 3-4 hours. The trays are then placed on ice, aspirated, rinsed once with cold binding rinse and incubated for one hour as above with 1 ml/well binding medium containing 0.5 ng/ml ^{125}I-PDGF. Labeling is terminated with 4 rinses of binding rinse and cell-associated ^{125}I-PDGF determined by extraction with solubilization buffer. Standard curves are obtained using 0, 0.05, 0.1, 0.2, 0.4, and 0.8 ng/ml purified PDGF and test samples compared to these values.

Results obtained by RRA for yeast C-8 eluates and 1X media samples are given in Table 2.

In addition, PDGF receptor binding by CM-sephadex media concentrates from yeast transformants containing plasmids pVSXm, pVS2αm, pVSBm, and pMPOT2 was compared to authentic PDGF. The results were interpreted by comparison to a standard curve generated with purified, unlabeled PDGF, as shown in Figure 13. Media from cultures transformed with the v-sis constructions are shown to compete with ^{125}I-PDGF for binding to the PDGF receptor. Media

from yeast cells transformed with pMPOT2 do not compete with radio-labeled PDGF for receptor binding.

D. Mitogenesis Assay

The ability of PDGF to stimulate DNA synthesis and cell growth in culture was the basis for its definition and discovery. ^{3}H-Thymidine incorporation into DNA of cultured cells responsive to PDGF (Raines and Ross, Meth. in Enz. 109: in press) is a preferred method for demonstrating the biological activity of PDGF-like molecules produced in yeast.

Test samples in 10mM acetic acid (100 ul/well) are added to quiescent cultures of mouse 3T3 cells in $2cm^2$ Costar 24-well culture dishes ($2-3x10^8$ cells/well in 1 ml). Quiescent test cultures can be obtained by plating the cells in 10% serum and allowing them to deplete the medium, 4-5 days. The test samples are removed from the wells at 20 hours and replaced with 0.5 ml of fresh medium per well containing 2 uCi/ml [^{3}H]-Thymidine and 5% (v/v) calf serum. After an additional 2-hour incubation at 37°C the cells are harvested by: aspirating off the medium, washing the wells twice each with 1 ml of ice-cold 5% TCA; solublizing TCA-insoluble material in 0.8 ml 0.25N NaOH with mixing; and counting 0.6 ml of this solution in 5 ml Aquasol in a liquid scintillation counter. Fold stimulation over control wells (100 ul of 10mM acetic acid alone) is determined, (normally 30-50 fold maximal stimulation) and compared to a standard curve obtained using purified PDGF preparations.

Table 2 presents results obtained in the mitogenesis assay for PDGF-like material produced in yeast and compares the activities of the PDGF-like material as measured by the above-described assay methods. Figure 12 depicts the mitogenic response elicited by concentrated

media from pl17-2 transformed E11-3c and pVSα transformed E8-11c compared to that obtained with purified human PDGF.

TABLE 2

Preparation	Transformant	ug/ml Protein	ng/ml PDGF by ELISA	RRA	MITOGENESIS
C-8 Eluates					
	pVSα/E8-11c	2.0	188	4.6	102
	pVS2α/E8-11c	16	864	16-97	310
	pl17-2/E11-3c	1.44	120	13.9	87
1X Media	pl17-2 E11-3c	--	4.2	0.18	2.5

In addition, the mitogenic response elicited by CM-sephadex concentrates from yeast transformants containing plasmids pVSαm, pVS2αm, pVSBm, and pMPOT2 was compared to that obtained with authentic PDGF. Referring to Figure 14, media from cultures transformed with the v-sis constructions stimulated uptake of ^{3}H-thymidine by quiescent 3T3 cells. As noted above, uptake of ^{3}H-thymidine by quiescent 3T3 cells is taken to be indicative of mitogenic stimulation. Media from yeast cells transformed with pMPOT2 showed no mitogenic activity.

The data presents clear evidence that growth media from the yeast strains constructed herein possess biological activities identical to authentic human PDGF. Further, these activities are readily detectable in nonconcentrated (1X) media from pl17-2 transformed strain E11-3c grown under POT1 selection.

From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit

and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

Claims

1. A DNA construct capable of directing the expression and secretion of biologically active PDGF analogs in eucaryotic cells, said DNA construct containing a transcriptional promoter followed downstream by a gene encoding a protein having substantially the same structure and/or mitogenic activity as PDGF, and a signal sequence directing the secretion of the protein from the eucaryotic cell.

2. The DNA construct of claim 1 wherein the eucaryotic cell is a yeast cell.

3. The DNA construct of claim 2 wherein said promoter is the yeast alpha-factor promoter or the yeast triose phosphate isomerase promoter.

4. The DNA construct of claim 2 wherein said promoter is followed downstream by the signal sequence of the yeast mating pheromone alpha-factor.

5. The DNA construct of claim 2 wherein said gene is followed downstream by a triose phosphate isomerase terminator.

6. The DNA construct of claim 1 wherein said gene is the v-sis gene of simian sarcoma virus or portions thereof encoding a protein having biological activity.

7. The DNA construct of claim 1 wherein said gene is a derivative of the v-sis gene of simian sarcoma virus or portions thereof encoding a protein having biological activity.

8. The DNA construct of claim 7 wherein the derivative of the v-sis gene of simian sarcoma virus is the portion of the v-sis gene which is substantially homologous to the B chain of PDGF.

9. The DNA construct of claim 1 wherein said gene is the human cDNA gene for PDGF or portions thereof encoding a protein having biological activity.

10. A method of preparing biologically active PDGF analogs comprising:

introducing into a eucaryotic host a DNA construct capable of directing the expression and secretion of biologically active PDGF analogs in eucaryotic cells, said DNA construct containing a transcriptional promoter followed downstream by a gene encoding a protein having substantially the same structure and/or mitogenic activity as PDGF, and a signal sequence capable of directing the secretion of the protein from the eucaryotic cell;

growing said eucaryotic host in an appropriate medium; and

isolating the protein product of said gene from said eucaryotic host.

11. The method of claim 10, including, after isolation of said protein product, purifying said product by gel filtration, exchange chromatography, or affinity chromatography.

12. The method of claim 10 wherein the eucaryotic cell is a yeast cell.

13. The method of claim 12 wherein said promoter is the yeast alpha-factor promoter or the yeast triose phosphate isomerase promoter.

14. The method of claim 12 wherein said promoter is followed downstream by the signal sequence of the yeast mating pheromone alpha-factor.

15. The method of claim 12 wherein said gene is followed downstream by a triose phosphate isomerase terminator.

16. The method of claim 10 wherein said gene is the v-sis gene of simian sarcoma virus or portions thereof encoding a protein having biological activity.

17. The method of claim 10 wherein said said gene is a derivative of the v-sis gene of simian sarcoma virus or portions thereof encoding a protein having biological activity.

18. The method of claim 17 wherein the derivative of the v-sis gene of simian sarcoma virus is the portion of the v-sis gene which is substantially homologous to the B chain of PDGF.

19. The method of claim 10 wherein said gene is the human cDNA gene for PDGF or portions thereof encoding a protein having biological activity.

20. The protein product produced by the method of claim 10.

21. A eucaryotic cell transformed with a DNA construct capable of directing the expression and secretion of biologically active PDGF analogs in eucaryotic cells, said DNA construct containing a transcriptional promotor followed downstream by a gene encoding a protein having substantially the same structure and/or mitogenic activity as PDGF, and a signal sequence capable of directing the secretion of the protein from the eucaryotic cell.

22. The eucaryotic cell of claim 21 wherein the eucaryotic cell is a yeast cell.

23. The yeast cell of claim 22 wherein said promoter is the yeast alpha-factor promoter or the yeast triose phosphate isomerase promoter.

24. The yeast cell of claim 22 wherein said promoter is followed downstream by the signal sequence of the yeast mating pheromone alpha-factor.

25. The yeast cell of claim 22 wherein said gene is followed downstream by a triose phosphate isomerase terminator.

26. The yeast cell of claim 22 wherein said DNA construct is the plasmid pll7-2.

27. The yeast cell of claim 22 wherein said DNA construct is the plasmid YEpVSX.

28. The yeast cell of claim 22 wherein said DNA construct is the plasmid YEpVS2X.

29. The eucaryotic cell of claim 21 wherein said gene is the v-sis gene of simian sarcoma virus or portions thereof encoding a protein having biological activity.

30. The eucaryotic cell of claim 21 wherein said gene is a derivative of the v-sis gene of simian sarcoma virus or portions thereof encoding a protein having biological activity.

31. The eucaryotic cell of claim 30 wherein the derivative of the v-sis gene of simian sarcoma virus is the portion of the v-sis gene which is substantially homologous to the B chain of PDGF.

32. The eucaryotic cell of claim 21 wherein said gene is the human cDNA gene for PDGF or portions thereof encoding a protein having biological activity.

33. The yeast cell of claim 22 wherein said DNA construct is the plasmid pVSBm.

34. A method of promoting the growth of mammalian cells, comprising incubating the cells with a biologically active PDGF analog expressed by a eucaryotic cell transformed with a DNA construct capable of directing the expression and secretion of biologically active PDGF analogs in eucaryotic cells, said DNA construct containing a transcriptional promoter followed downstream by a gene encoding a protein having substantially the same structure and/or mitogenic activity as PDGF, and a signal sequence capable of directing the secretion of the protein from the eucaryotic cell.

35. The method of claim 34 wherein said growth is carried out in vitro.

36. The method of claim 34 wherein said cells are normal cells or transformed cells.

37. The method of claim 34 wherein said eucaryotic cell is a yeast cell.

38. The method of claim 37 wherein said promoter is the yeast alpha-factor promoter or the yeast triose phosphate isomerase promoter.

39. The method of claim 37 wherein said promoter is followed downstream by the signal sequence of the yeast mating pheromone alpha-factor.

40. The method of claim 37 wherein said gene is followed downstream by a triose phosphate isomerase terminator.

41. The method of claim 34 wherein said gene is the v-sis gene of simian sarcoma virus or portions thereof encoding a protein having biological activity.

42. The method of claim 34 wherein said gene is a derivative of the v-sis gene of simian sarcoma virus or portions thereof encoding a protein having biological activity.

43. The method of claim 42 wherein the derivative of the v-sis gene of simian sarcoma virus is the portion of the v-sis gene which is substantially homologous to the B chain of PDGF.

44. The method of claim 34 wherein said gene is the human cDNA gene for PDGF or portions thereof encoding a protein having biological activity.

45. A DNA construct capable of replication in yeast and containing the yeast triose phosphate isomerase promoter, said yeast promoter being followed downstream by the signal sequence of the yeast mating pheromone alpha-factor, said signal sequence being followed downstream respectively by the portion of the v-sis gene which is substantially homologous to the B chain of PDGF and a triose phosphate isomerase terminator.

46. The plasmid pll7-2.

47. The plasmid YEPVS$^{\alpha}$.

48. The plasmid YEPVS2$^{\alpha}$.

49. The plasmid pVSB.

50. The plasmid pVSBm.

51. A DNA construct capable of directing the expression and secretion of biologically active PDGF analogs in eucaryotic cells, said DNA construct containing a transcriptional promoter followed downstream by the portion of the v-sis

gene which is substantially homologous to the B chain of PDGF, said portion encoding a protein having substantially the same structure and/or mitogenic activity as PDGF, and a signal sequence directing the secretion of the protein from the eucaryotic cell.

52. A method of preparing biologically active PDGF analogs, comprising:

introducing into a eucaryotic host a DNA construct capable of directing the expression and secretion of biologically active PDGF analogs in eucaryotic cells, said DNA construct containing a transcriptional promoter followed downstream by the portion of the v-sis gene which is substantially homologous to the B chain of PDGF, said portion encoding a protein having substantially the same structure and/or mitogenic activity as PDGF, and a signal sequence directing the secretion of the protein from the eucaryotic cell;

growing said eucaryotic host in an appropriate medium; and

isolating the protein product of said gene from said eucaryotic host.

53. A eucaryotic cell transformed with a DNA construct capable of directing the expression and secretion of biologically active PDGF analogs in eucaryotic cells, said DNA construct containing a transcriptional promoter followed downstream by the portion of the v-sis gene which is substantially homologous to the B chain of PDGF, said portion encoding a protein having substantially the same structure and/or mitogenic activity as PDGF, and a signal sequence directing the secretion of the protein from the eucaryotic cell.

54. A method of promoting the growth of mammalian cells, comprising incubating the cells with a biologically active PDGF analog expressed by a eucaryotic cell transformed with a DNA construct capable of directing the expression and secretion of biologically active PDGF analogs in eucaryotic

cells, said DNA construct containing a transcriptional promoter followed downstream by the portion of the v-sis gene which is substantially homologous tc the B chain of PDGF, said portion encoding a protein having substantially the same structure and/or mitogenic activity as PDGF, and a signal sequence directing the secretion of the protein from the eucaryotic cell.

EXPRESSION OF BIOLOGICALLY ACTIVE PDGF ANALOGS IN EUCARYOTIC CELLS

Abstract of the Disclosure

Biologically active PDGF analogs expressed in eucaryotic cells are disclosed. The analogs are produced by yeast strains transformed with an extrachromosomal element composed of a strong transcriptional promoter directing the expression of a gene which encodes a protein having substantially the same biological activity as PDGF. Suitable genes include the v-sis gene or a derivative of the v-sis gene of simian sarcoma virus or portions thereof, or the human cDNA gene for PDGF or portions thereof. In particular, DNA sequences encoding polypeptides substantially homologous to the B chain of PDGF are preferred. A secretory signal sequence may be provided upstream of the gene, enabling secretion of the gene product from the host cell. Mitogenic activity is one of the biological activities possessed by these PDGF analogs, making them useful in promoting the growth of mammalian cells.

705,175

I certify that on the date specified below, this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Commissioner of Patents and Trademarks, Washington, D.C. 20231.

4/18/85
Date

David J. Mahl

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Mark J. Murray et al.

Serial No. : 705,175

Filing Date : February 25, 1985

For : EXPRESSION OF BIOLOGICALLY ACTIVE PDGF ANALOGS IN EUCARYOTIC CELLS

Examiner :

Art Unit :

Docket : 9908.410 CIP

Date : April 18, 1985

Commissioner of Patents and Trademarks

Washington, D.C. 20231

Attention: Application Division

Sir:

Enclosed is a Declaration and Power of Attorney and a Verified Statement for U.S.S.N. 705,175, filed February 25, 1985, for a EXPRESSION OF BIOLOGICALLY ACTIVE PDGF ANALOGS IN EUCARYOTIC CELLS.

We are also enclosing our check No. 18008 in the amount of $535.00 for the filing fee. Because the applicant qualifies for small entity status, the fee has been calculated as follows:

Basic Fee:	$150.00
Total Claims (54, 34 extra)	170.00
Ind. Claims (14, 11 extra)	165.00
Surcharge:	50.00
TOTAL:	$535.00

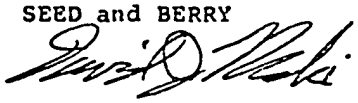
04/30/85 705175 1 201 150.00 CK
04/30/85 705175 1 202 165.00 CK
04/30/85 705175 1 203 170.00 CK
1 205 50.00 CK

The Commissioner is authorized to charge any additional filing fees or to credit any overpayment to Deposit Account No. 19-1090.

Respectfully submitted,

Mark J. Murray et al.

SEED and BERRY



David J. Maki

Registration No. 31,392

DJM:pc

Enclosures: Declaration and Power of Attorney

Verified Statement

Check

(206) 622-4900

DECLARATION AND POWER OF ATTORNEY

As the below-named inventors, we hereby declare that:

Our residences, post office addresses, and citizenships are as stated below under our names.

We verily believe we are the original, first and joint inventors of an invention entitled "EXPRESSION OF BIOLOGICALLY ACTIVE PDGF ANALOGS IN EUCARYOTIC CELLS," which is described and claimed in the specification and claims of patent application Serial No. 705,175 which we filed in the United States Patent and Trademark Office on February 25, 1985; that this application (hereinafter referred to as "later application") in part discloses and claims subject matter disclosed in our earlier filed pending application entitled "EXPRESSION OF BIOLOGICALLY ACTIVE PLATELET DERIVED GROWTH FACTOR ANALOGS IN YEAST," Serial No. 660,496, filed October 12, 1984 (hereinafter referred to as "earlier application").

We acknowledge our duty to disclose information of which we are aware which is material to the examination of this application.

We hereby appoint RICHARD W. SEED, Registration No. 16,557; BENJAMIN F. BERRY, Registration No. 15,525; ROBERT J. BAYNHAM, Registration No. 22,846; EDWARD W. BULCHIS, Registration No. 26,847; GEORGE C. RONDEAU, JR., Registration No. 28,893; DAVID H. DEITS, Registration No. 28,066; WILLIAM O. FERRON, JR., Registration No. 30,633; and DAVID J. MAKI, Registration No. 31,392, composing the firm of SEED and BERRY, 1001 Bank of California Center, Seattle, Washington 98164, our attorneys to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. Please direct all telephone calls to David J. Maki at (206) 622-4900.

We further declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that the making of willfully false statements and the like is punishable

by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and may jeopardize the validity any patent issuing from this patent application.

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant or Patentee: Mark J. Murray et al. Attorney's Docket No. 9908.410CIP
Serial or Patent No.: 705,175
Filed or Issued: February 25, 1985
For: EXPRESSION OF BIOLOGICALLY ACTIVE PDGF ANALOGS IN EUCARYOTIC CELLS

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS
(37 CFR 1.9(f) and 1.27(b)) -- INDEPENDENT INVENTOR

As a below named inventor, I declare that I qualify as an independent inventor as defined in 37 CFR 1.9(c) for purposes of paying reduced fees under Section 41(a) and (b) of Title 35, United States Code, to the Patent and Trademark Office with regard to the invention entitled EXPRESSION OF BIOLOGICALLY ACTIVE PDGF ANALOGS IN EUCARYOTIC CELLS described in

[ ] the specification filed herewith
[x] application Serial No. 705,175 filed 2/25/85
[ ] patent No. _____, issued _____

I have not assigned, granted, conveyed or licensed and am under no obligation under contract or law to assign, grant, convey or license, any rights in the invention to any person who could not be classified as an independent inventor under 37 CFR 1.9(c) if that person had made the invention, or to any concern which would not qualify as a small business concern under 35 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

Each person, concern or organization to which I have assigned, granted, conveyed or licensed or am under an obligation under contract or law to assign, grant, convey or license any rights in the invention is listed below:

[x] no such person, concern or organization
[ ] persons, concerns or organizations listed below*

*NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

FULL NAME _____

ADDRESS _____

[ ] individual

[ ] small business concern

[ ] nonprofit organization

FULL NAME _____

ADDRESS _____

[ ] individual

[ ] small business concern

[ ] nonprofit organization

FULL NAME _____

ADDRESS _____

[ ] individual

[ ] small business concern

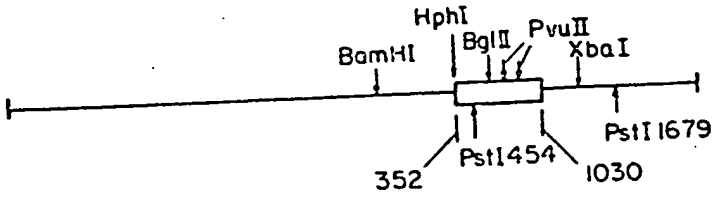
[ ] nonprofit organization

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earlier of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b).

I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Mark J. Murray	James Darrel Kelly	
NAME OF INVENTOR	NAME OF INVENTOR	NAME OF INVENTOR
Signature of Inventor	Signature of Inventor	Signature of Inventor
4-16-85	4/16/85	
Date	Date	Date

FIG. 1A



v-sis-helper viral junction

Hph I

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                                  367                             382                             397
CT ATG ACC CTC ACC TGG CAG GGG GAC CCC ATT CCT GAG GAG CTC TAT AAG ATG
   MET Thr Leu Thr Trp Gln Gly Asp Pro Ile Pro Glu Glu Leu Tyr Lys MET

                                                                                                 Pst I
                                                                                                 457
          412                             427                             442                   |
CTG AGT GGC CAC TCG ATT CGC TCC TTC AAT GAC CTC CAG CGC CTG CTG CAG GGA
Leu Ser Gly His Ser Ile Arg Ser Phe Asn Asp Leu Gln Arg Leu Leu Gln Gly

                                  472                             487                             502
GAG TCC GGA AAA GAA GAT GGG GCT GAG CTG GAC CTG AAC ATG ACC CGC TCC CAT
Asp Ser Gly Lys Glu Asp Gly Ala Glu Leu Asp Leu Asn MET Thr Arg Ser His

                                                                                                 562
     517                             532                             547                        |
TCT GGT GGC GAG CTG GAG AGC TTG GCT CGT GGG AAA AGG AGC CTG GGT TCC CTG
Ser Gly Gly Glu Leu Glu Ser Leu Ala Arg Gly Lys Arg Ser Leu Gly Ser Leu

                                                                                                 607
                577                             592                                            |
AGC GTT GCC CAG CCA GCC ATG ATT GCC GAG TGC AAG ACA CGA ACC GAG GTG TTC
Ser Val Ala Glu Pro Ala MET Ile Ala Glu Cys Lys Thr Arg Thr Glu Val Phe

  Bgl II                                                                                         667
  622                             637                             652                           |
GAG ATC TCC CGG CGC CTC ATC GAC CGC ACC AAT GCC AAC TTC CTG GTG TGG CCG
Glu Ile Ser Arg Arg Leu Ile Asp Arg Thr Asn Ala Asn Phe Leu Val Trp Pro
```

FIG. 1B

```
      682                 697                 712                 727
CCC TGC GTG GAG GTG CAG CGC TGC TCC GGC TGT TGC AAC AAC CGC AAC GTG CAG
Pro Cys Val Glu Val Gln Arg Cys Ser Gly Cys Cys Asn Asn Arg Asn Val Gln

                        Pvu II
            742         |   757                 772
TGC CGG CCC ACC CAA GTG CAG CTG CGG CCA GTC CAG GTG AGA AAG ATC GAG ATT
Cys Arg Pro Thr Gln Val Gln Leu Arg Pro Val Gln Val Arg Lys Ile Glu Ile

   787                 802                 817                 832
GTG CGG AAG AAG CCA ATC TTT AAG AAG GCC ACG GTG ACG CTG GAG GAC CAC CTG
Val Arg Lys Lys Pro Ile Phe Lys Lys Ala Thr Val Thr Leu Glu Asp His Leu

                       Pvu II
           847         |862                 877
GCA TGC AAG TGT GAG ATA GTG GCA GCT GCA CGG GCT GTG ACC CGA AGC CCG GGG
Ala Cys Lys Cys Glu Ile Val Ala Ala Ala Arg Ala Val Thr Arg Ser Pro Gly

892                 907                 922                 937
ACT TCC CAG GAG CAG CGA GCC AAA ACG ACC CAA AGT CGG GTG ACC ATC CGG ACG
Thr Ser Gln Glu Gln Arg Ala Lys Thr Thr Gln Ser Arg Val Thr Ile Arg Thr

      952                 967                 982                 997
GTG CGA GTC CGC CGG CCC CCC AAG GGC AAG CAC CGG AAA TGC AAG CAC ACG CAT
Val Arg Val Arg Arg Pro Pro Lys Gly Lys His Arg Lys Cys Lys His Thr His

            1012                1027                1043      1053
GAC AAG ACG GCA CTG AAG GAG ACC CTC GGA GCC TAA GGGCATCGGC AGGAGAATAT
Asp Lys Thr Ala Leu Lys Glu Thr Leu Gly Ala

     1063      1073      1083      1093      1103      1113      1123
GGGCAGCGGG TCTCCTGCCA GCGGCCTCCA GCATCTTGCC CAGCAGCTCA AGAAGAGAAA AAAGGACTGA

     1133      1143      1153      1163      1173      1183      1193
ACTCCACCAC CATCTTCTTC CCTTAACTCC AAAAACTTGA AATAAGAGTG TGAAAGAGAC TGATAGGGTC

     1203      1213      1223      1233      1243      1253      1263
GCTGTTTGAA AAAAACTGGC TCCTTCCTCT GCACCTGGCC TGGGCCACAC CCAAGTGCTG TGGACTGGCC

     1273      1283      1293      1303      1313      1323      1333
CGAGGGGCCC TGCACGTGGC CCTGAGCACC TCTCAGTGTA GCCTGCCTGG TCCCTAGACC CCTGGCCAGC

                                          XbaI || v-sis-helper viral junction
     1343      1353      1363      1373      |
TCCAAGGGGA GGCACCTCCA GGCAGGCCAG GCTACCTCGG GGGTCTAG
```

FIG. 1B

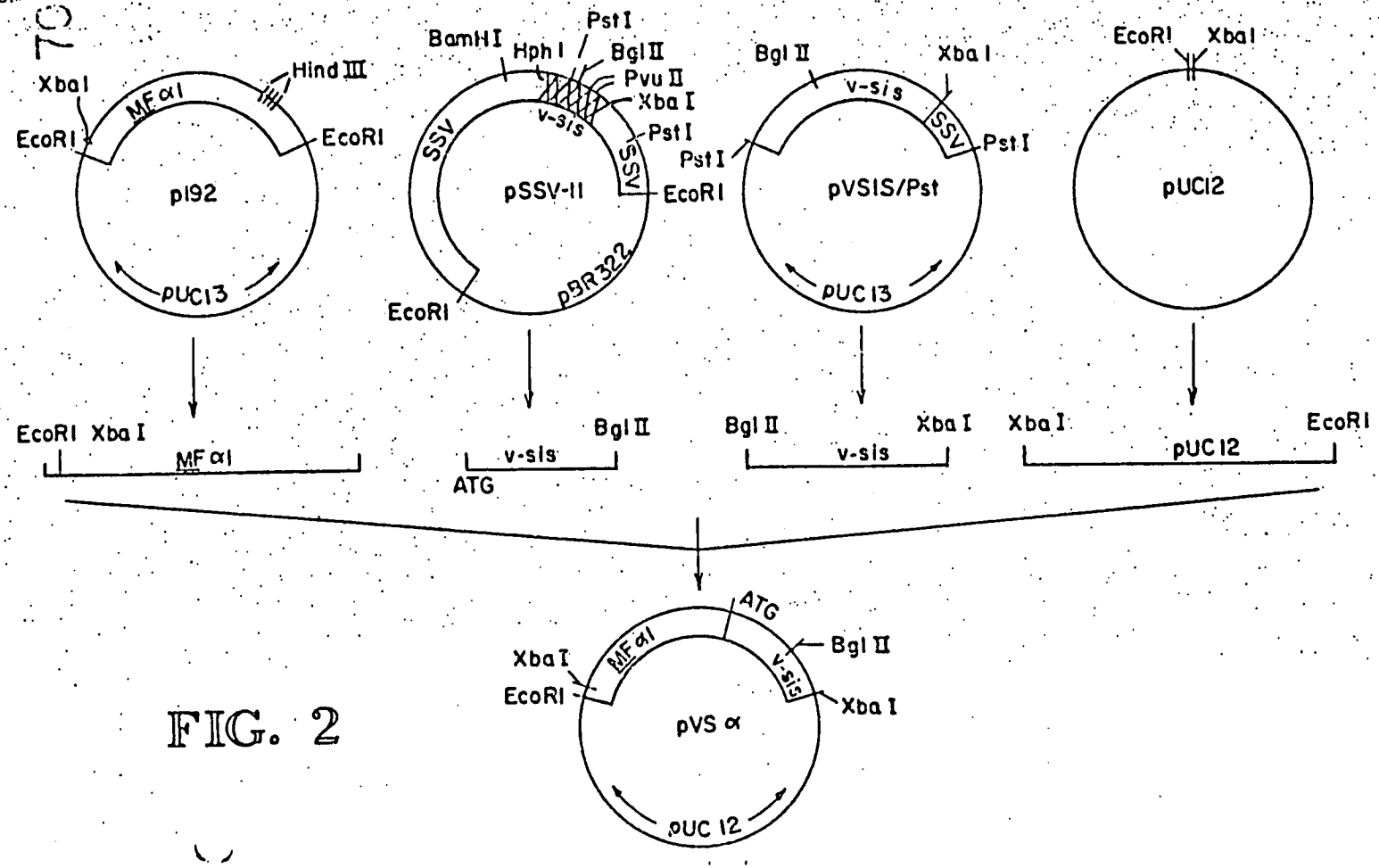
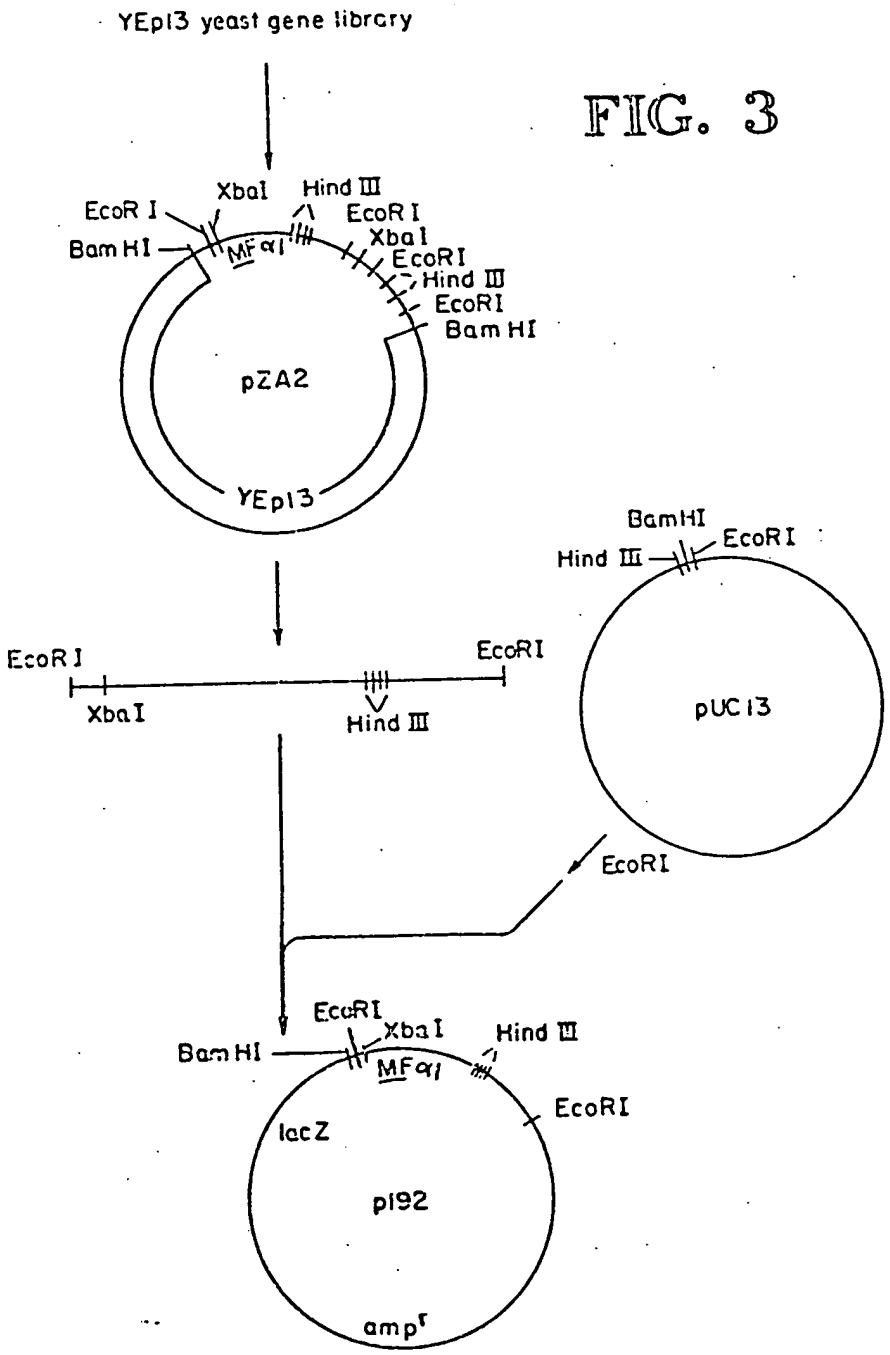

Xba I
MFα1
Hind III
EcoRI
EcoRI
p192
pUC13
BamHI
Hph I
Pst I
Bgl II
Pvu II
Xba I
Pst I
EcoRI
SSV
v-sis
SSV
pSSV-11
pBR322
EcoRI
Bgl II
Xba I
v-sis
SSV
Pst I
Pst I
pVSIS/Pst
pUC13
EcoRI
Xba I
pUC12
EcoRI Xba I
MFα1
Bgl II
v-sis
ATG
Bgl II
Xba I
v-sis
Xba I
EcoRI
pUC12
ATG
Xba I
MFα1
Bgl II
v-sis
EcoRI
Xba I
pVSα
pUC 12

FIG. 2

FIG. 3


YEp13 yeast gene library
EcoR I
Xba I
Hind III
EcoR I
Bam HI
MFα1
Xba I
EcoRI
Hind III
EcoRI
Bam HI
pZA2
YEp13
BamHI
Hind III
EcoRI
pUC13
EcoRI
Xba I
Hind III
EcoRI
EcoRI
Xba I
Hind III
Bam HI
MFα1
EcoRI
lacZ
p192
ampr

705175

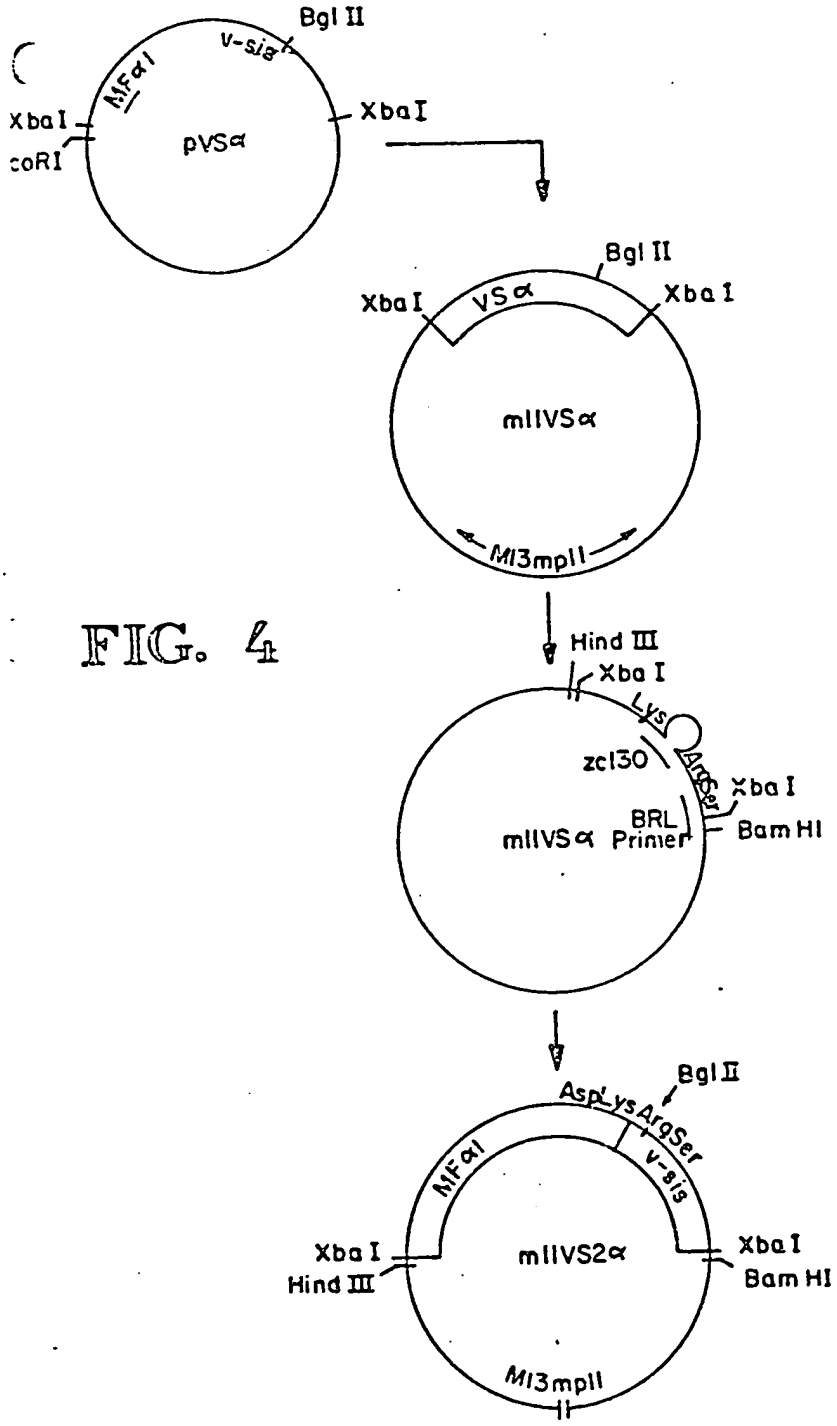

Bgl II
v-sig
MFα1
Xba I
coR I
pVSα
Xba I
Bgl II
Xba I
VSα
Xba I
mIIVSα
M13mpII
Hind III
Xba I
Lys
zc130
ArgSer
Xba I
BRL
Primer
mIIVSα
Bam HI
Asp Lys ArgSer
Bgl II
MFα1
v-sig
Xba I
Hind III
mIIVS2α
Xba I
Bam HI
M13mpII

FIG. 4

705175

FIG. 5

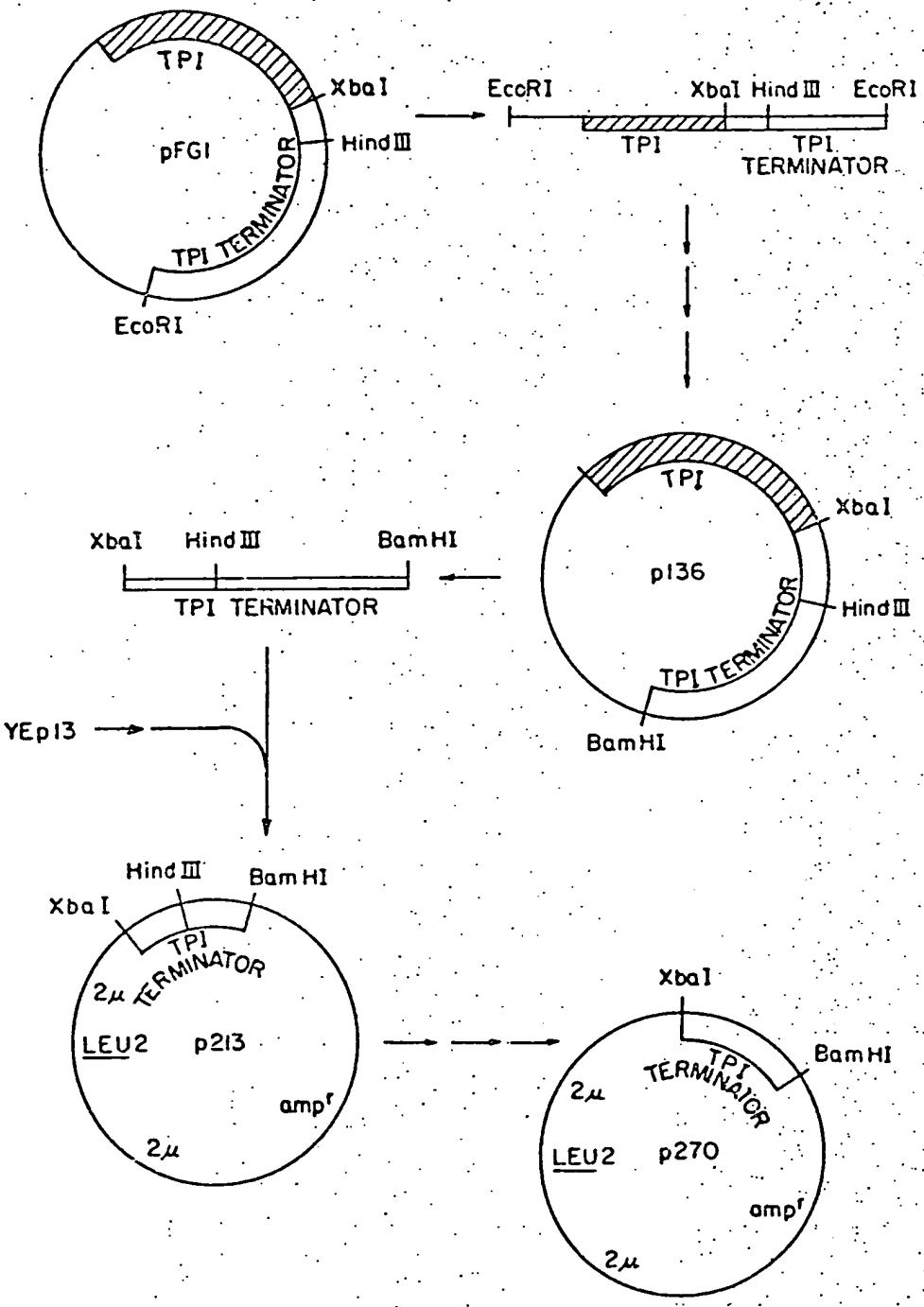
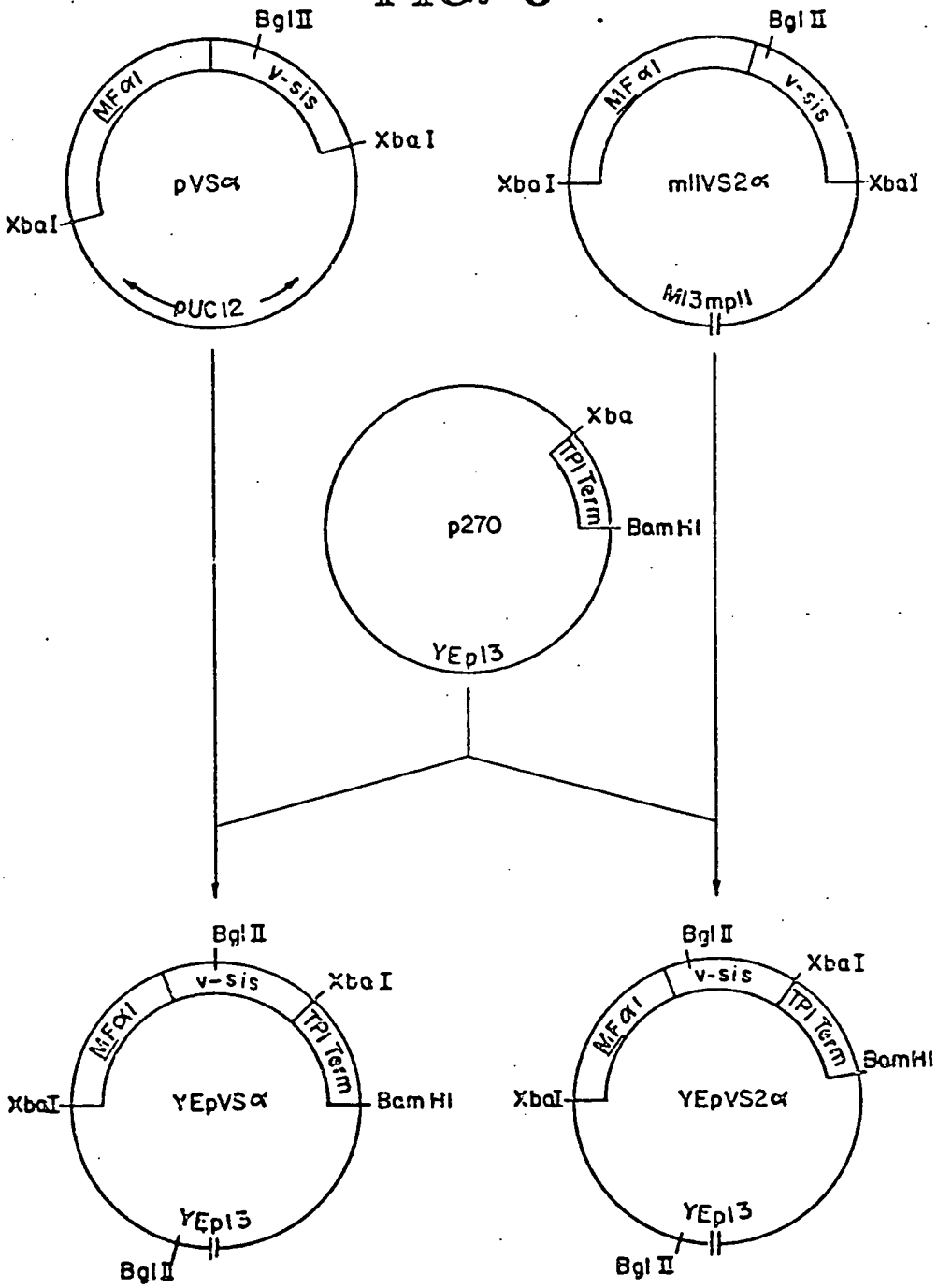

TPI
XbaI
pFGI
HindIII
TPI TERMINATOR
EcoRI
EcoRI
XbaI HindIII
EcoRI
TPI
TPI TERMINATOR
TPI
XbaI
p136
HindIII
TPI TERMINATOR
BamHI
XbaI
HindIII
BamHI
TPI TERMINATOR
YEp13
HindIII
BamHI
XbaI
TPI TERMINATOR
2μ
LEU2
p213
ampr
2μ
XbaI
TPI TERMINATOR
BamHI
2μ
LEU2
p270
ampr
2μ

FIG. 6


BglII
MFα1
v-sis
XbaI
pVSα
XbaI
pUC12
BglII
MFα1
v-sis
XbaI
mIIVS2α
XbaI
M13mpII
Xba
TPI Term
p270
BamHI
YEp13
BglII
v-sis
XbaI
MFα1
TPI Term
XbaI
YEpVSα
BamHI
YEp13
BglII
BglII
v-sis
XbaI
MFα1
TPI Term
BamHI
XbaI
YEpVS2α
YEp13
BglII

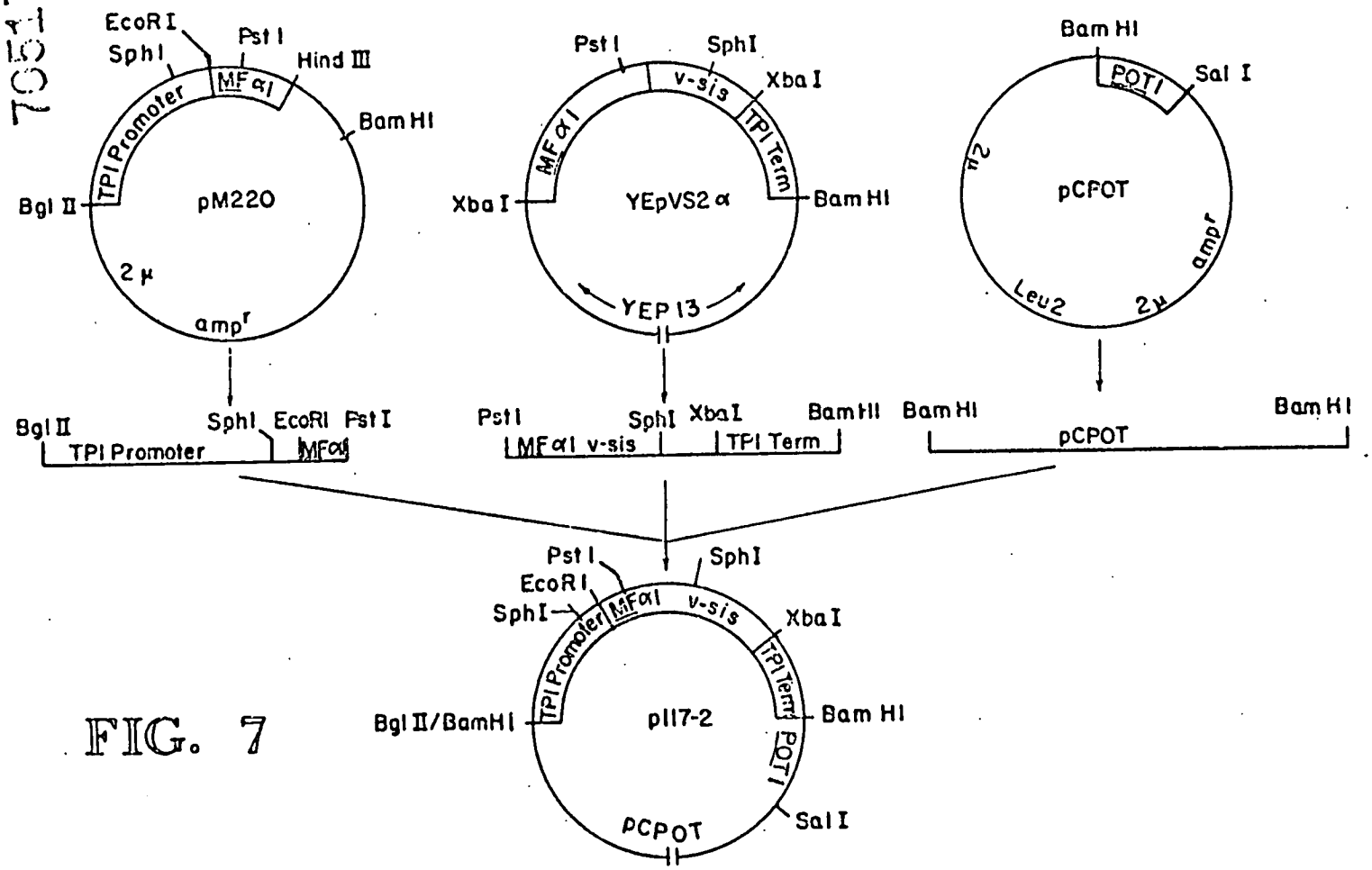
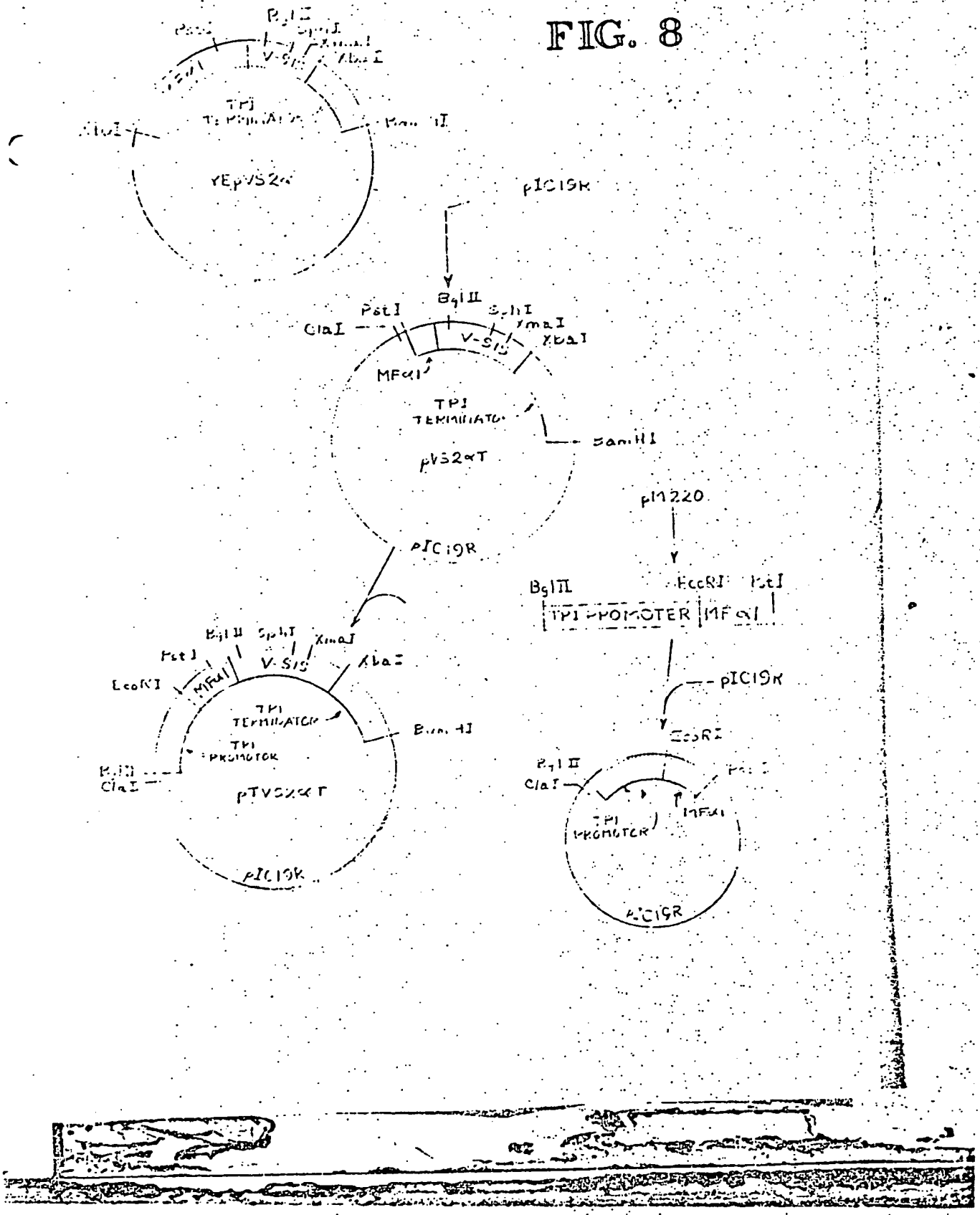


FIG. 7

FIG. 8


YEpVS2α
pICI9R
PstI
BglII
SphI
XmaI
XbaI
ClaI
V-S1S
MFα1
TPI TERMINATOR
HamHI
pVS2αT
pM220
BglII
EcoRI
PstI
TPI PROMOTER
MFα1
pICI9R
EcoRI
BglII
ClaI
TPI PROMOTER
MFα1
pICI9R

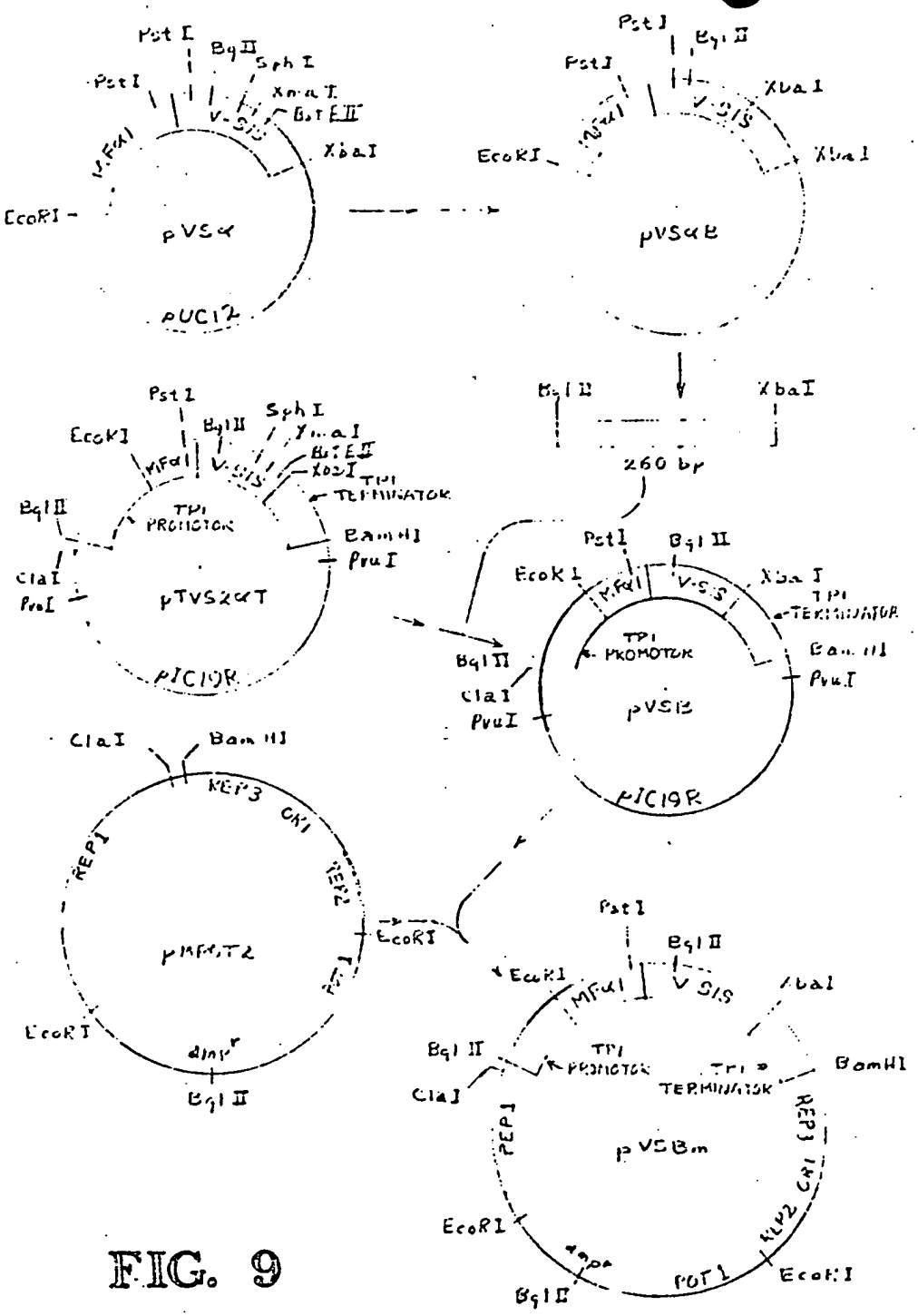

Pst I
Bgl II
Sph I
Xma I
Bst EII
Pst I
MFα1
V-S/S
Xba I
EcoRI
pVSα
pUC12
pVSαB
260 bp
TPI PROMOTOR
TPI TERMINATOR
Bam HI
Pvu I
Cla I
pTVS2αT
pIC19R
pVSB
REP3
CAT
REP2
REP1
pMRM.T2
EcoRI
ampr
Bgl II
pVSBm
POT1
KLP2

FIG. 9

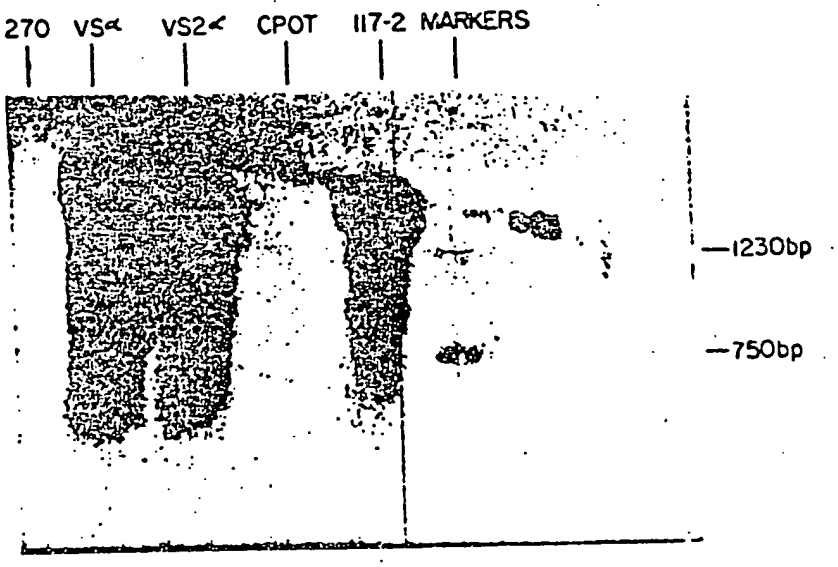


FIG. 10

FIG. 11

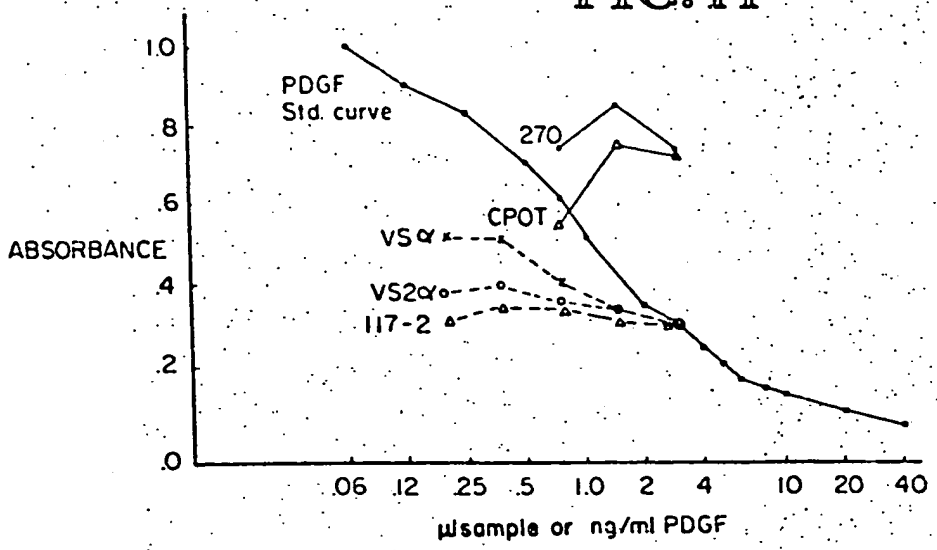

PDGF
Std. curve
270
CPOT
VSα
VS2α
117-2
ABSORBANCE
1.0
.8
.6
.4
.2
.0
.06 .12 .25 .5 1.0 2 4 10 20 40
µl sample or ng/ml PDGF

FIG. 12

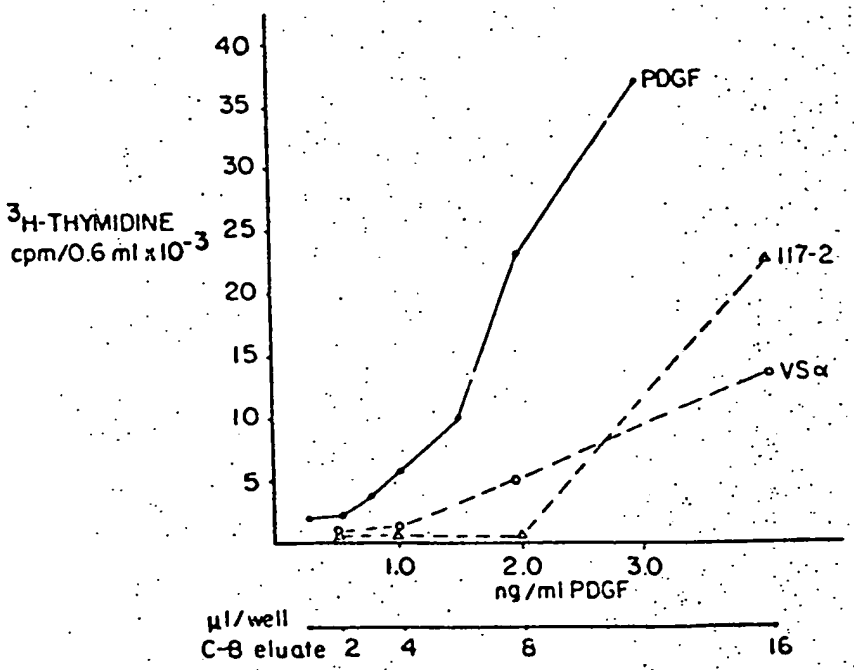

^{3}H-THYMIDINE
cpm/0.6 ml x 10^{-3}
40
35
30
25
20
15
10
5
PDGF
117-2
VSα
1.0
2.0
3.0
ng/ml PDGF
µl/well
C-8 eluate 2 4 8 16

FIG. 13

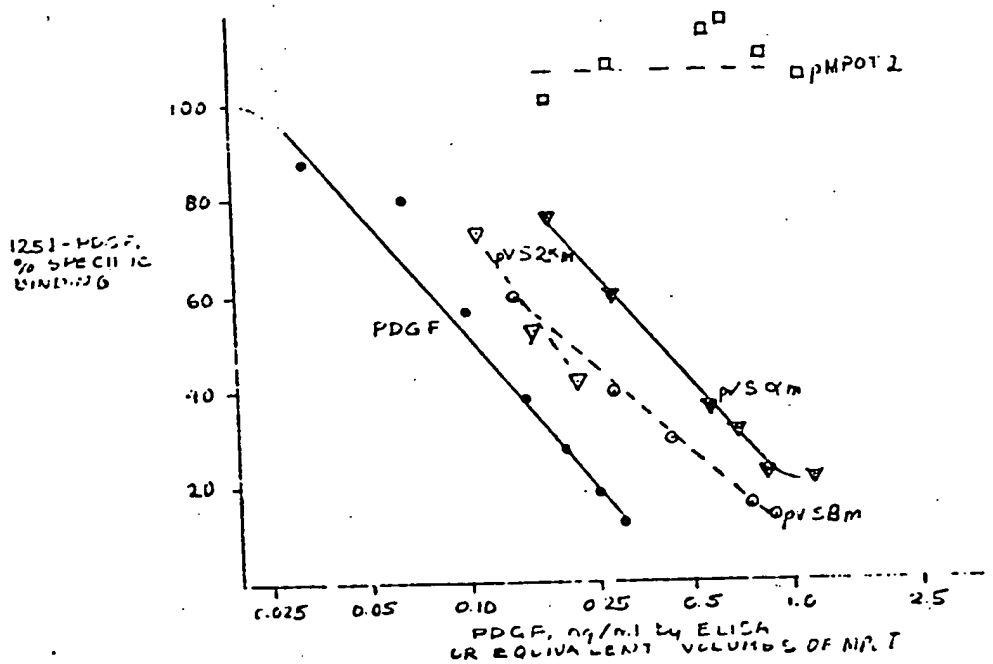
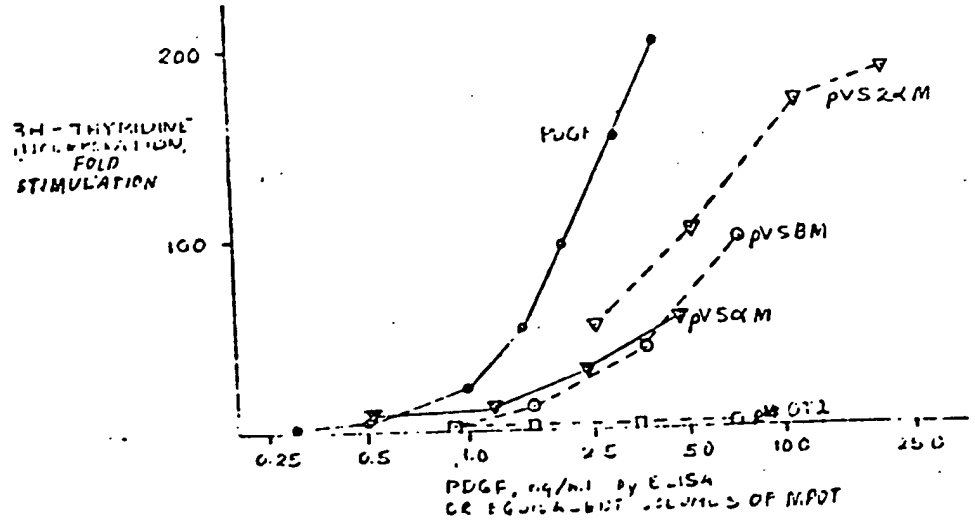

pMPOT 2
100
80
60
40
20
125I-PDGF, % SPECIFIC BINDING
pVS2αM
PDGF
pVSαm
pVSBm
0.025
0.05
0.10
0.25
0.5
1.0
2.5
PDGF, ng/ml by ELISA OR EQUIVALENT VOLUMES OF MPOT

FIG. 14


200
100
3H-THYMIDINE INCORPORATION, FOLD STIMULATION
PDGF
pVS2αM
pVSBM
pVSαM
0.25
0.5
1.0
2.5
5.0
10.0
25.0
PDGF, ng/ml by ELISA OR EQUIVALENT VOLUMES OF MPOT